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MOLECULAR EPIDEMIOLOGY STUDIES ON PSEUDOMONAS AERUGINOSA
ISOLATES FROM PATIENTS WITH CYSTIC FIBROSIS

BY

QIMING SUN



A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND
RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF..Master of Science.....

DEPARTMENT OF .Biochemistry.....

EDMONTON, ALBERTA, CANADA

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ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen which readily infects hosts who have been immunocompromised by cancer, thermal injury, or cystic fibrosis (CF). Polar pili, produced by this organism, are filamentous appendages which have been shown to be virulence factors by virtue of their ability to promote adherence to epithelial tissue and thus promote colonization.

Studies of the epidemiology of P. aeruginosa in patients with CF were carried out. A total of 32 clinical isolates from CF patients were examined using a method called restriction fragment length polymorphism (RFLP). Five unique RFLP patterns were observed. A further study of five serially collected P. aeruginosa isolates (CD2,3,4,5 and 10) from a single CF patient revealed genetic identity and stability among those strains which appeared to have different colonial morphology, serotyping and antibiograms.

A second set of serially collected P. aeruginosa isolates from a CF patient, the GA group, consisted of six strains in which pilins displayed different molecular weights and unique intrastrain immunological patterns when examined in whole-cell immunoblotting experiments. The pilin genes and their flanking regions of GA1 and GA2, were cloned and sequenced at the nucleotide level and found to be identical. Analysis of the sequence of this 2179-bp cloned DNA fragment revealed that the upstream region of the pilin DNA sequence had a strong

homology with the NifA recognition sites normally located in the upstream region of nitrogen fixation genes. Three inverted repeats found in the 3'-flanking sequence may be responsible for the termination of mRNA transcription. Pilin gene expression and phage sensitivity assays were also carried out and suggested that one or more cryptic factors may be involved in producing the unique immunological pilin phenotypes in the GA1 and GA2 strains of P. aeruginosa.

Eight serologically related or unrelated isolates of P. aeruginosa pilin peptide sequences were analyzed: PAK, PAO, CD, P1, K122-4, KB7, 492C and GA1&2 (GA). The comparative studies of the characteristics of the pilin peptides, homology alignments, hydropathic indices, hydrophilicity profiles, secondary structure predictions and antigenicity predictions were carried out using PC/GENE computer programs.

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ABBREVIATIONS

A	adenine
AA	autoagglutinable
Ala	alanine
Arg	argnine
Asn	asparagine
Asp	aspartate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
CF	cystic fibrosis
Cys	cysteine
d	2'-deoxyribo
DNA	deoxyribonucleic acid
ELISA	enzyme linked immunosorbant assay
G	guanine
Gln	glutamine
Glu	glutamate
Gly	glycine
HC	highly conserved
His	histidine
HV	hypervariable
Ile	isoleucine
IPTG	isopropyl B-D-thiogalacto-pyranoside
Kb	kilobase pair
L.B.	Luria broth
Leu	leucine
LPS	lipopolysaccharide
Lys	lysine
MC	moderately conserved
Met	methionine
mRNA	messenger RNA
NA	not applicable
NMePhe	N-methylphenylalanine
PAGE	polyacrylamide gel electrophoresis
Phe	phenylalanine
PIA	<u>Pseudomonas</u> isolation agar
<u>PilA</u>	gene encoding the structural subunit of pilin
Pro	proline
PT	polytypeable

RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
Ser	serine
SDS	sodium dodecyl sulfate
T	thymine
Thr	threonine
Trp	tryptophan
Tyr	tyrosine
Val	valine
X-gal	5-bromo-4-chloro-3-indolyl-B-D-galacto-pyranoside

CHAPTER I

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic pathogen capable of causing lethal infections in patients weakened by cystic fibrosis (CF), leukemia, tissue damage caused by severe burns, cancer and other immunosuppressive illness (Bodey et al., 1983). Factors which are involved in P. aeruginosa virulence are lipopolysaccharide (LPS) (Pollack et al., 1979) alginate (Woods et al., 1985), exotoxin A (Iglewski et al., 1977), exoenzyme S (Iglewski et al., 1978), various proteases (Moriyama, 1964), hemolysin (Johnson et al., 1980), phospholipase C (Berk et al., 1977), outer membrane proteins (Gilleland et al., 1984; Mutharia et al., 1983) and polar pili, which are filamentous extracellular organelles (Woods et al., 1980; Paranchych and Frost, 1988). These polar pili are involved in such processes as adherence of bacteria to mammalian epithelial cells (Punsalang and Sawyer, 1973; Stephens and McGee, 1981; Trust et al., Woods et al., 1980; Rothbard et al., 1985), and a primitive form of surface translocation known as twitching motility (Henrichsen, 1973). They also act as the receptors for a number of pilus-specific bacteriophages (Bradley et al., 1974) including spherical RNA phages which attach to the lateral surface of the pilus (Bradley, 1972a), filamentous single-stranded DNA phages which attach to the

pilus tip (Bradley, 1973), and a variety of double-stranded phages with contractile tails which attach to the lateral surface of the pilus (Bradley and Pitt, 1974).

P. aeruginosa polar pili are chromosomally encoded and have diameters of 5.2 nm and an average length of 2500nm (Bradley, 1972b; Folkhard et al., 1981). They consist of identical pilin subunits, which range in size from approximately 15,000-18,000 daltons (Robertson et al., 1977; Hermodson et al., 1978; Watts et al., 1983a; Sastry et al., 1983; Schoolink et al., 1984; Marrs et al., 1985; Haas and Meyer, 1986). Hundreds of pilin subunits are assembled in a helical array of five subunits per turn (Watts et al., 1983a), with each turn having a 4.1 nm pitch (Paranchych and Frost, 1988).

It has been shown that Pseudomonas pili belong to a class of pili which are present in a wide range of gram-negative bacteria including Neisseria gonorrhoeae, Neisseria meningitidis, Moraxella bovis, Moraxella nonliquefaciens, and Bacteroides nodosus (Elleman and Hoyne, 1984; Froholm and Sletten, 1977; Marrs et al., 1985; Paranchych and Frost, 1988; Perry et al., 1987; Schoolink et al., 1984). The pili of these species are characterized by: i) a highly conserved sequence of 30 hydrophobic amino acids at the N-terminus; ii) the loss of a 6-7 residue positively charged amino acid leader peptide from the N-terminal region of the pilin precursor and iii) methylation of the resulting amino terminus

to yield N-methylphenylalanine. Therefore, they are generally referred to as NMePhe pili (Paranchych and Frost, 1988).

A. STRUCTURE-FUNCTION RELATIONSHIPS OF NMePhe PILIN

The pilus protein has been found to promote important functions. Special regions of the polypeptide chain are responsible for anchoring pilin to membranes, transporting pilin across membranes, interacting with various accessory proteins involved in pilus assembly, or subunit-subunit interactions. The surface-exposed regions of the pilus protein are often immunologically active or function as a recognition site for pilus-specific bacteriophages or mammalian cell receptors (Paranchych and Frost, 1988; Paranchych, 1990). Examination of some amino acid sequences from different genera that encode NMePhe pili have shown several significant features.

First, every NMePhe pilin sequence has a highly conserved N-terminus, which consists of 30 amino acids including a short positively charged leader sequence. This propilin has strong similarities to the typical signal sequences required by proteins destined for the periplasm or the outer membrane of gram-negative bacteria. However, regular signal sequences have a polar C-terminal region after the stretch of hydrophobic amino acids which marks the site of cleavage (von Heijne, 1985). NMePhe propilin is unique in that it lacks such a region recognized by the signal peptidase. Instead, processing

occurs just after the positively charged N-terminus and the mature pilin subunit maintains its N-terminal hydrophobic sequence. It has been suggested that this region may play an important role in pili morphogenesis, with accessory factors involved in the pilus assembly system and subunit-subunit interactions (Mattick *et al.*, 1987). These conclusions were based on observations that Tyr residues at position 24 and 27 of *P. aeruginosa* PAK pilin are completely buried in intact pili, but absolutely exposed when the pili are dissociated into dimeric subunits with the non-ionic detergent octyl glucoside (Watts *et al.*, 1983a; Mattick *et al.*, 1987). More recent studies have shown that only the first 36 amino acids of the propilin are required for membrane integration and that the C-terminal region of propilin may associate with the N-terminal part to stabilize the region between residues 30-65 against degradative processes (Pasloske *et al.*, 1988a). The predicted secondary structure of this hydrophobic N-terminal domain exhibits high potential for both alpha-helix and beta-sheet structure. This may indicate that the conformation of the amino terminal portion of the molecule is governed by environmental conditions: alpha-helical in assembled pili and beta-sheet during synthesis and transport into the membranes (Steiner *et al.*, 1980; Briggs *et al.*, 1986). Secondly, the NMePhe pilins contain at least two cysteine residues which usually exist at the C-terminus of the pilin protein. It has been determined that these cysteine residues are in their

oxidized state and contribute to the formation of an intrachain disulfide bridge (Schoolnik et al., 1984; Sastry et al., 1985). Most of the P. aeruginosa pilins (PAK, PAO, CD group, PA103, P1), the M. bovis pilins and N. gonorrhoeae pilins have a disulfide loop near the C-terminus, in which the spacing varies from 12 to 30 amino acid residues between the two Cys residues (Sastry et al., 1983; Elleman, 1988; Schoolnik et al., 1984). B. nodosus pilins have a large 40-residue region between Cys residues in the central region of the protein. P. aeruginosa strain K122-4 has both the large central region as well as the C-terminal disulfide loop. Another B. nodosus pilin is characterized by a small, 9-residue disulfide loop near the end of the conserved N-terminal region, and a 12-residue disulfide loop at the C-terminus (Elleman et al., 1986; Elleman, 1988).

Studies on P. aeruginosa and N. gonorrhoeae pilins have shown a relationship between the disulfide loop structure and the adherence capability of these strains. It has been found that a proteolytically derived peptide from P. aeruginosa pilin, encompassing the disulfide loop, was able to inhibit the adherence of the homologous bacterial cell (Paranchych et al., 1986). With N. gonorrhoeae, it has also been demonstrated that type-specific monoclonal anti-pili serum directed to the disulfide loop was able to inhibit the adherence of purified pili to human buccal epithelial cells and they were able to protect epithelial cells against

homologous gonococci in a cytotoxic assay (Nicolson et al., 1987). More interestingly, recent work revealed that antipeptide antibodies against a 17-residue oxidized peptide which contained the disulfide loop sequence were able to cross-react with both homologous and heterologous pili (Lee et al., 1989a). Also, monoclonal antibody and antipeptide Fab fragments against the disulfide loop region of P. aeruginosa strain PAK pilin were shown to prevent adherence of the homologous strain from human buccal epithelial cells (Doig et al., 1990; Lee et al., 1989b). More recently, it was observed that a Ser-to-Asn mutation within the area of the disulfide loop region in P. aeruginosa PAK pilin conferred resistance to a filamentous, pilus tip-binding phage Pf (Pasloske and Paranchych, unpublished observations). Collectively, these results provided strong evidence that the disulfide loop region may be exposed at the pilus surface to act as the receptor-binding domain which recognizes epithelial cell receptors.

B. IMMUNOLOGICAL STUDIES OF NMpHe PILIN

The antigenic regions of the P. aeruginosa strain PAK pilus protein were determined by Watts et al. (1983b). Arginine-specific cleavage was used for the initial fragmentation of the pilus polypeptide into four large peptides (CTI, CTII, CTIII, CTIV). The various peptides were subjected to immunoblot and direct ELISA assays, as well as

competitive ELISA experiments. Four distinct epitopes were identified: one weak cross-reactive epitope in the N-terminal region, a moderately immunogenic epitope between residues 70-81, a strongly immunogenic epitope in region 82-110, and another weak epitope in the C-terminal region between residues 128-144. More recently, antibodies were raised against synthetic peptides representing predicted potential surface regions of the PAK pilin and employed in a variety of immunological studies with intact pilin to identify the surface domains of the native pilus. The studies identified five immunogenic regions, including the disulfide loop region (Lee *et al.*, 1989a, 1989b). Rabbit antiserum raised against an analog of the disulfide loop region with Cys-129 substituted by an Ala residue bound weakly to PAK pilin in direct ELISA experiments suggesting the importance of the disulfide bridge in preparing antipeptide antibodies that bind to the native protein in this region (Lee *et al.*, 1989a). These immunological studies revealed useful information about the protein structure of intact pili, and suggested that the disulfide loop region of pilin may be a good candidate for a Pseudomonas vaccine.

C. ANTIGENIC VARIATIONS

Broadly defined, antigenic variation refers to the ability of a microbe to alter the antigenic characters of its surface components. The ability of a microbe to undergo

antigenic variation is defined here as the ability of a single strain to express different antigenic variants of a cellular component. Many bacterial species show interstrain variations of surface antigenicity, which is believed to be due to antigenic drift, or the slow accumulation of mutations in the structural genes encoding the surface components. In contrast, some bacteria can produce intrastrain serologically distinguishable surface determinants due to a biphasic transition resulting from a rapid on-and-off switching of gene expression modulated by recombination at the DNA level and DNA inversion events (Seifert and So, 1988).

Antigenic variations of some Pseudomonas pilins have been observed and studied. One extensively studied example revealed the mechanism of pilin variation in N. gonorrhoeae pili. In vitro studies have shown that a single cell of a gonococcal strain is capable of giving rise to many different progeny which produce pilins with new epitopes (Hagblom et al., 1985). In strain MS11, there is one locus (pilE1) responsible for the expression of pilin. Another locus, pilS1, is a 3-kilobase region of DNA composed of six variant pilin sequences. Antigenic variation occurs when a copy of a variant gene in a silent pil locus is recombined into an expression site, thus giving rise to a new antigenic pilin (Steven and So, 1988). Unlike N. gonorrhoeae, B. nodosus has a single copy of the pilin gene per genome (Elleman, 1988), and the serotypes found were considered to be the result of antigenic drift. In M.

bovis strain EPP63, inversion of a 2-kilobase chromosomal DNA encoding the pilin sequence results in the alternate expression of one or the other of two different pilin subunits: alpha and beta subunits (Marrs et al., 1988). However, the mechanism by which antigenic variation arises in single pilin gene systems of P. aeruginosa is still unknown. It seems likely that the various pilin serotypes have arisen via antigenic drift and/or recombination. It is often assumed that pathogenic microbes have evolved antigenic variation for the sole purpose of evading the host immune response and modulating their host range, as well as adaptation to hostile environments.

D. PILIN EXPRESSION, TRANSLOCATION AND PROCESSING

Proteins destined for export within prokaryotes are usually nascently expressed with an extra 15 to 30 residues at the N-terminus known as a signal sequence (Michaelis and Beckwith, 1982). The signal extension contains one or more positively charged residues, followed by a hydrophobic region and then a site of cleavage which is slightly polar (von Heijine, 1985). As mentioned earlier, Pseudomonas pilin has a shorter leader sequence, and the pilin is not fully mature until it has been methylated at the N-terminal Phe residue. This particular modification appears to be a relatively unique attribute of NMePhe pilins. According to the studies carried out from deletion mutagenesis, chemical mutagenesis,

transposon mutagenesis and site-specific mutagenesis, it has been suggested that the NMePhe pilins' maturation involves a two-step process requiring two unique enzymes for the cleavage and the methylation, and the mode of pilin secretion may follow an unusual pathway (Paranchych and Frost, 1988; Pasloske et al., 1988a; Pasloske and Paranchych, 1988).

Studies demonstrated that the expression of the cloned pilin gene of *P. aeruginosa* PAK within *E. coli* mini cells produced both unprocessed propilin and processed pilin which were located mainly in the cytoplasmic membrane (Finlay et al., 1986). To define regions of the subunit necessary for its stability and transport within *E. coli*, deletion mutants of the pilin gene of *P. aeruginosa* PAK were constructed using a SP6/T7 promotor system (Pasloske et al., 1988a). The results showed that a mutant having an Ile-4 to Met-7 deletion was stably expressed and pilin protein was primarily transported to the cytoplasmic membranes of *E. coli* in the unprocessed form. It was also found that only the first 36 amino acids of the unprocessed pilin subunit were capable of becoming associated with the *E. coli* membrane. When the cloned mutant pilin genes from PAK were expressed in *P. aeruginosa* PAO1, it was found that the foregoing deletion mutant was capable of partitioning the pilin fragment into the membrane as efficiently as normal pilin (Pasloske and Paranchych, 1988).

Most interestingly, when a cloned pilin gene containing a Glu-5-to-Lys mutation was expressed in *P. aeruginosa* PAO1,

the mutation did not prevent leader sequence removal but did inhibit the methylation of the N-terminal phenylalanine (Pasloske and Paranchych, 1988). Furthermore, the mutant pilin was found to be incorporated into heterogeneous mixed pili with host PAO1 wild-type pilin subunits, but it was unable to polymerize as a homopolymer (Pasloske *et al.*, 1989a). This suggested that the NMePhe, or Glu-5, or both amino acids play a key role in pilin polymerization. The inability of the modified pilin to assemble in the *Pseudomonas* host suggested that the deleted residues are required either for recognition by the cleavage and assembly machinery or for stabilization of the subunit-subunit interactions.

E. *PSEUDOMONAS* PILIATION AND REGULATION OF PILIN GENE EXPRESSION

Pseudomonas pili have been shown to play a necessary role in the adhesion of the bacterium during infection (Woods *et al.*, 1980a; Reichert *et al.*, 1983), but they also provide antigenic targets for the host immune system and are thus a liability to the bacterium. In response to these two opposing pressures, two types of variation in piliation occur: interstrain/intrastrain antigenic variation (see Antigenic Variations section above) and phase variation. Phase variation involves the switching of pilated cells to the nonpilated state and has been observed in *N. gonorrhoeae* (Swanson and Barrera, 1983) and *B. nodosus* (Skerman *et al.*, 1981). In *N.*

gonorrhoeae, this variation is accomplished both by regulating the production of the pilin and by its state of assembly (Swanson et al., 1985). The regulation of piliation in P. aeruginosa strain PAK was carried out using the pilus-specific bacteriophage PO4 to isolate spontaneous pilus variants (Johnson and Lory, 1987). Hyperpilated mutants which overproduce pilin mRNA were found. It has been suggested that pilus retraction is a component of a pathway involved in the feedback regulation of pilin structural gene pilA transcription. This pathway could sense a demand for pilA transcription through a depletion of the inner membrane pool of pilin or some other pilus assembly proteins. The pool size of these proteins could be regulated by the rate at which the pilus assembles and disassembles. Therefore, a lack of pilus retraction would lead to a fluctuation in the levels of these proteins and increased pilA transcription (Johnson and Lory, 1987).

Studies on the regulation of NMePhe pilin gene expression have revealed that pilin genes from Pseudomonas strains are controlled by NtrA-dependent promoters. NtrA, the gene product of ntrA, is an alternative, 54-kilodalton sigma factor initially discovered to be involved in the transcription of the genes which fix and assimilate nitrogen in organisms such as Klebsiella pneumoniae and E. coli (Gussin et al., 1986). NtrA recognizes an atypical consensus sequence of CTGGYAYRN, TTGCA, extending from position -26 to -10 relative

to the transcription initiation site (TIS) (Gussin et al., 1986). The NtrA-dependent promoters in the enteric or nitrogen fixing organisms are activated in the presence of a second regulatory gene product from either ntrC(NtrC) or ntrA(NtrA) which binds to sequences upstream of the NtrA promoter (Johnson et al., 1986). The pilin promoter region at -10 and -35 base pairs from the TIS shows no significant homology to the consensus E. coli promoter, but the -12 and -24 regions show a high degree of homology to promoters which require the ntrA gene product for transcription. Several other Pseudomonas promoters and the promoter of the homologous pilin genes from other bacterial species were also found to share the homology to this sequence (Johnson et al., 1986). More recently, a putative regulatory sequence 115 base pairs upstream of the TIS was noted (Pasloske et al., 1988b) as having homology to the NifA binding sequence of K. pneumoniae which has the consensus sequence TGTN₁₀ACA (Buck et al., 1986). Further determination of the functional relevance of this sequence was carried out by comparing the levels of pilin expression and quantitative measurements of the promoter strengths of DNA fragments with or without this NifA-like sequence called sipR (Pasloske, et al., 1989b). It was found that the deletion of a 48-kilobase fragment containing the sipR sequence resulted in much lower pilin and pilus production and the promoter activity dropped by 90%. These results indicated that a region of DNA upstream of the NtrA-like pilin gene promoter operates

as a positive regulatory sequence affecting pilin expression.

F. MOLECULAR EPIDEMIOLOGY OF PSEUDOMONAS AERUGINOSA

P.aeruginosa has a peculiar propensity to colonize and infect the respiratory tract of patients with cystic fibrosis (CF) (Woods, et al., 1976). Although many studies on P. aeruginosa have been carried out in the areas of biochemistry, immunology and molecular biology, epidemiological studies of P. aeruginosa in patients with CF have been strongly impeded by the existing typing methods such as colonial morphology, antimicrobial susceptibility, pyocin or phage typing, and O-serotyping (Bobo et al., 1973; Bradley and Pitt, 1974; Goven and Gillies, 1969; Penketh et al., 1983; Thomassen et al., 1979). These procedures may be unreliable because of the inherent phenotypic variability of P. aeruginosa strains (Ogle et al., 1987). For example, the serotyping method is based upon an agglutination reaction between bacterial lipopolysaccharide (LPS) and type-specific rabbit antisera (Fisher et al., 1969). This serotyping scheme works well for clinical isolates of P. aeruginosa with possess smooth LPSS (Pier and Thomas, 1982). However, CF isolates of P. aeruginosa are different, the majority are rough LPS strains, deficient in the O-polysaccharide side chain which is responsible for the serotyping reaction (Hancock et al., 1983). These strains are therefore non-typable with commercial antisera directed against LPS (Fisher et al., 1969). Antimicrobial suscepti-

bility often changes under the pressure of antibiotic therapy (Gilbert 1985), limiting the usefulness of antibiotic resistance profiles as a typing method (Thomassen *et al.*, 1979). Outer membrane proteins and analysis of their profiles therefore can not be used for epidemiological purposes (Mutharia *et al.*, 1982). These conventional typing methods probably do not offer the discriminatory power necessary to distinguish among isolates and provide answers to basic epidemiological questions.

In 1987, Ogle and co-workers (Ogle *et al.*, 1987) used a DNA probe as an epidemiological marker for *P. aeruginosa*. It is a 741-base-pair PstI-NruI fragment derived from the upstream region of the exotoxin A gene. The probe was used to hybridize to certain restriction enzyme digests of *P. aeruginosa* chromosomal DNA. Serial *P. aeruginosa* isolates from individual CF patients which varied in colonial morphology, serotype and biotype were found to be identical when analyzed with this probe. On the other hand, indistinguishable isolates cultured from unrelated patients were distinguishable by using a DNA probe method. Thus, a DNA probe was able to eliminate much of the ambiguity associated with trying to establish relations among clinical isolates by means of colonial morphology. This DNA probe typing method is also called restriction fragment length polymorphism (RFLP) typing.

By using the similar techniques, Paranchych *et al.* (Paranchych and Frost, 1988) have confirmed many of the

observations of Ogle et al with a different gene probe: a 1.2 kb HindIII fragment containing the PAK pilin gene (Pasloske et al., 1985). This probe was used to group over 500 *P. aeruginosa* isolates from patients with CF, burns and cancers. In these studies, 21 different restriction fragment patterns were classified, whereas pilin gene cloning and sequencing studies revealed 7 unique pilin genes among several hundred isolates characterized thus far (Paranchych et al., unpublished observations). Of the 500 isolates screened, many were serial isolates from individual patients, and in most instances, the restriction fragment patterns were of only one type, despite apparent differences in O serotype, colonial morphology and antibiotic patterns. Serial isolates differing with respect to colonial morphology and serotype were further studied and found to have identical pilin gene sequences (Pasloske et al., 1988d; also see this thesis chapter III).

More recently, Speert et al used the same probe and RFLP typing method to screen 249 isolates and found 21 different RFLP types (Speert et al., 1989). They also observed that there was substantial phenotypic heterogeneity among CF isolates of *P. aeruginosa* from individual sputum samples, and that the serotype frequently changed in isolates from serial samples of the same patient. Multiple sequential isolates from individual patients revealed co-colonization with more than one type in some patients.

G. THESIS OBJECTIVES

As discussed in the foregoing, biochemistry and molecular biology studies have revealed significant information about Pseudomonas NMePhe pili with regard to their subunit structure, protein and DNA sequence, mechanism of gene expression and regulation, antigenic variation, immunological properties, as well as pilin secretion and processing. However, there are still many unanswered questions relating to the mechanisms of pathogenesis of P. aeruginosa, particularly with regard to targetting of virulence factors as potential vaccine candidates.

This thesis is focused on the following areas: i) characterization of clinical isolates of P. aeruginosa by using pilin gene DNA and anti-pilin antibodies as probes; ii) cloning, sequencing and expression of the two P. aeruginosa isolates GA1 and GA2; iii) comparison of the pilin proteins and DNA sequences of several Pseudomonas NMePhe pili-producing strains.

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Chapter II

MATERIALS AND METHODS

A. Materials

1. Strains, Plasmids, Growth Media, and Antibiotics used

All of the bacterial strains other than Pseudomonas aeruginosa isolates, plasmids and bacteriophage are listed in Table II-1. The P. aeruginosa isolates from CF patients are listed in Table III-1 (Chapter III). P. aeruginosa strains PAK and PAO were from Dr. D.E. Brandley, Memorial University, St. Johns, Newfoundland. Strain P1 was obtained from Dr. D.P. Speert, University of British Columbia, Vancouver, B.C.

The media used were Luria-Bertani (L-Broth) medium (Maniatis et al., 1985), TSB agar and Pseudomonas isolation agar (PIA).

L-Broth consisted of 1% (w/v) tryptone (DIFCO Laboratories, Detroit, Michigan) and 1% (w/v) NaCl, pH 7.2. TSB agar and Pseudomonas isolation agar (PIA) were purchased from DIFCO. The final antibiotic concentrations were as follows: ampicillin, chloramphenicol and kanamycin, 50 µg/ml; mercuric chloride (HgCl_2), 15 µg/ml; carbenicillin, 100 µg/ml and 300 µg/ml; tetracycline 10 µg/ml and 150 µg/ml for Escherichia coli and Pseudomonas aeruginosa, respectively. When the cells were grown in L-Broth liquid culture, the

antibiotic concentrations were half of the above concentration.

2. Animals

Female New Zealand white rabbits, obtained from Ellerslie Animal Farms, University of Alberta, Edmonton, Alberta, were used for the production of polyclonal anti-pilin antisera. The rabbits were between 6 weeks and 2 years of age during the various injections and bleedings.

3. Buffers and Solutions

All solutions were prepared from analytical grade or molecular biology grade reagents in double distilled water unless otherwise stated.

- 1) TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
- 2) TBE buffer (10X): 1 M Tris-base, 0.83M Boric acid, 10 mM EDTA, pH 8.3.
- 3) SSC (20X) solution: 149 NaCl, 88g Na-Citrate, pH 7.0 and make up volume to 1 liter with H₂O.
- 4) Protein gel electrode buffer (10X): 250mM Tris-base, 192mM Glycine, 0.1% SDS (w/v).
- 5) Immunoblot transfer tank buffer: 24 mM Tris-base, 192 mM Glycine, 20% Methanol (v/v), pH 8.3.
- 6) Immunoblot buffer I: 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, 0.25% Gelatin (w/v). 0.05% Nonidet P40 (v/v), pH 7.4, make up volume with H₂O.

- 7) Immunoblot buffer II: 1 M NaCl, 5 mM EDTA, 50 mM Tris-HCl, 0.25% Gelatin (w/v), 0.40% Sakosyl (w/v), pH 7.4, make up volume with H₂O.
- 8) Southern blot solution I: 500 ml H₂O containing 10.8 ml of concentrated HCl.
- 9) Southern blot solution II: 10 ml of 10 N NaOH, 17.5 g NaCl, make up to 500 ml with H₂O.
- 10) Southern blot solution III: 60.5 g Tris-base, 17.5 g NaCl, pH to 7-7.5 with concentrated HCl and make up to 500 ml with H₂O.
- 11) ELISA coating buffer: 0.5 M sodium bicarbonate, pH 9.6.
- 12) PBS buffer: 20 g NaCl, 0.5 g KH₂PO₄, 1.875 g Na₂HPO₄ · 7H₂O, 0.5 g KCl, make up volume to 2.5 liters with H₂O.
- 13) PBS.T buffer: PBS buffer containing 0.05 (v/v) Tween 20.
- 14) PBS.T.BSA: PBS.T buffer containing 1% (w/v) Bovine serum albumin (BSA).
- 15) Protein polyacrylamide gel staining solution: 5 g Coomassie Blue R-250, 25% Isopropanol, 10% Acetic acid make up to 1 liter with H₂O.
- 16) Protein gel destaining solution: 10% Acetic acid, 10% Methanol in H₂O.
- 17) Sequencing gel soaking solution: 10% Acetic acid, 12% Methanol.

- 18) Nick translation buffer (10 X): 50 μ l of 1 M Tris(pH 7.8), 0.1 M MgCl_2 , 1 μ l of 2 mg/40 μ l BSA, 0.7 μ l of mercaptoethanol, 49 μ l of H_2O .

4. Chemicals and Reagents

Acrylamide, N,N'-methylenebisacrylamide (electrophoresis grade) and EDTA were obtained from BDH Chemicals, Toronto. Agarose (electrophoresis grade) was purchased from Bethesda Research Laboratories (BRL), Gaithersburg. Ultra pure Tris, urea and sodium dodecylsulfate (SDS) were purchased from Schwarz/Mann Biotech, Cleveland. CsCl was obtained from Pharmacia, Sweden. Ampicillin, chloramphenicol, kanamycin, mercuric chloride, carbenicillin, tetracycline, isopropyl B-D-thiogalacto-pyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-B-D-galacto-pyranoside (X-gal), bovine serum albumin (BSA), nonidet P40 (an octyl-phenol-ethylene-oxide condensate containing an average of 9 ethylene oxide per molecule), sarcosyl (97% n-lauryl-sarcosine) were all purchased from SIGMA, St. Louis. Freund's complete and incomplete adjuvant were obtained from DIFCO, Detroit.

5. Radioactive materials

Protein A [^{125}I], 25 $\mu\text{Ci}/\text{ml}$ in 0.03M phosphate buffer containing 40% ethanol and acetic acid (pH 4.0), [^{32}P]-labeled or [^{35}S]-labeled nucleotide triphosphate α -[^{32}P]dATP and α -[^{35}S]dATP were purchased from New England Nuclear Corp., Dupont

Canada, Lachine, Quebec.

6. Enzymes

All restriction endonucleases, such as BamHI, ClaI, EcoRI, HincII, HindIII, PstI, Sau3A and DNA polymerase I, Klenow large fragment were purchased from Bethesda Research Laboratories (BRL). DNase and Calf intestine alkaline phosphatase were purchased from Boehringer Mannheim, Germany. RNase and lysozyme were purchased from SIGMA.

7. DNA Sequencing Kit

A single stranded-DNA sequencing kit SEQUENASE was purchased from United States Biochemical Corp., Cleveland.

B. METHODS

1. Chromosome DNA Purification

Chromosomal DNA from *P. aeruginosa* was prepared using a modification of the procedure of Coleman et al (Coleman et al., 1983). 40 ml bacterial overnight cultures were centrifuged (5000 rpm, 5 minutes) and the sedimented bacteria were resuspended in 2 ml 25 mM Tris-HCl (pH 8.0) buffer and 6 ml volume of the solution containing 10 mM Tris-HCl/1 mM EDTA/20% sucrose (wt/vol) (pH 8.0), then left on ice 10 minutes. 4 mg lysozyme was added, the solution was thoroughly mixed using a vortex apparatus, then kept on ice for another 10 minutes. After the addition of 160 μ l 10% SDS, the solution was mixed again and kept on ice for 10 more minutes. The solution was left at room temperature for 5 minutes and at 70°C for an additional 5 minutes. Eight ml of TE buffer was added and the lysate was passed several times through a 10 ml syringe with a 16 gauge needle to mix the solution and shear the DNA (thus lowering the viscosity of the DNA). Next, the lysate was mixed with phenol (saturated with TE) three times (centrifuged at 7000 rpm at 20°C for 5 minutes each time). The supernatant solution was mixed with the RNase solution to a final concentration of 100 μ g/ml and left at room temperature for 30 minutes. The phenol extraction was continued until the protein interface disappeared. One tenth volume of 3 M Sodium acetate and 3X volumes of 95% cold (4°C) ethanol were added to

the supernatant solution and the tube contents were mixed by inverting several times. The DNA clumps were removed with a glass hook and dissolved in 7 ml of TE buffer. 1.05 ml of 3 M Sodium acetate was then added. The solution was extracted with phenol once more and 18 ml of 95% ethanol was added to precipitate the DNA. The solution was centrifuged at 10,000 rpm for 20 minutes. The DNA pellet was dried and resuspended in 1 to 2 ml of TE buffer and the DNA concentration was measured.

2. CLONING OF DNA INSERTS

All the protocols listed below were performed according to Maniatis et al (1982) and method guidelines from Boehringer Mannheim GmbH Biochemica and Promega.

I) Preparation of Vector and Insert DNA for Cloning

Both fragment and vector DNA were digested with appropriate restriction enzymes to generate compatible ends for cloning. When a single restriction enzyme was used to prepare the vector, the DNA was treated with calf intestinal alkaline phosphatase to remove 5'phosphate groups and thus to prevent recircularization of the vector during ligation.

a. Standard reaction mixture:

2-5 μ g DNA

2 μ l appropriate 10X restriction enzyme buffer

5-25 u appropriate restriction enzyme

H₂O was added to 20 μ l final volume

- b. The mixture was incubated at 37°C (or other appropriate temperature) for 2 hours.
- c. The reaction was tested to determine its completion by electrophoresis of a sample (0.3 μ g) on a 0.9-1.0% agarose minigel.
- d. The DNA digests were then extracted with phenol and the DNA was precipitated with ethanol and resuspended in TE buffer.

II) Ligation of Vector and Insert DNA

It is recommended that a 1:1, 1:3 or a 3:1 molar ratio of vector : insert be used when cloning a fragment into a vector.

a. Ligation reaction mixture:

appropriate vector and insert DNA

1 unit T₄ DNA ligase

5 μ l 10X ligase buffer

H₂O was added to 50 μ l

- b. Ligation reactions were incubated at room temperature for 2 hours.
- c. Following the ligation reaction, plasmid DNA were transformed into competent cells of an appropriate host strain.

III) Preparation of Competent Cells

The procedure used was according to Maniatis et al (1982).

The desired bacterial host strain was grown in L. Broth medium with vigorous shaking at 37°C overnight. 100 ml of L. Broth cell culture was inoculated with 1 ml of the overnight culture, and the cells were grown until the O.D.₆₀₀ was about 0.2 to 0.5. The culture was chilled on ice for 10 minutes. The cell suspension was centrifuged at 5000 rpm for 5 minutes at 4°C. The cells were resuspended in half of the original culture volume with an ice-cold, sterile solution of 50 mM CaCl₂ and 10 mM Tris.HCl (pH 8.0) and placed on ice for 20 minutes. The suspension was again centrifuged at 5000 rpm for 5 minutes and the cells were resuspended in 1/15 of the original volume of an ice-cold, sterile solution of 50 mM CaCl₂ and 10 mM Tris.HCl (pH 8.0). The cells were stored at 4°C for 12-24 hours.

IV) Transformation of Plasmids into Competent Cells and Selection of transformants

Two hundred µl of desired host competent cells were mixed with 50 µl ligation mixture and incubated on ice for 45 minutes. Next, the cells were heat shocked by transferring the tube to a 42°C waterbath for 1-2 minutes. One ml of L. Broth was added and the mixture was incubated for 60 minutes at 37°C. To use the blue/white color screening for recombinants, the transformed E. coli cells were placed on LB plates containing 100 µg/ml ampicillin, 0.5 mM IPTG and 40

$\mu\text{g/ml}$ BCIG (X-Gal). The plates were incubated overnight at 37°C .

V) Colony Hybridization

This procedure was based on Maniatis et al (1982).

A nitrocellulose filter was placed onto an agar plate containing the selective antibiotic. Sterile toothpicks were used to transfer the individual bacterial colonies to be screened onto the filter and then onto a master agar plate that contained the selective antibiotic but no filter. Each colony was streaked in an identical position on both plates. The plates were incubated at 37°C until the colonies had grown to 0.5-1.0 mm in width. The filter was lifted and the filter and the master plate were marked in an asymmetric manner.

The filters (colony side up) were placed on SDS-impregnated 3MM paper for 3 minutes. Next, the filters were transferred to a second sheet of 3 MM paper that had saturated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) and left there for 5 minutes. The filters were then transferred to a third sheet of 3MM paper that had been saturated with neutralizing solution (1.5 M NaCl, 0.5 M Tris.HCl, pH 8.0) and left for 5 minutes. The filters were baked for 2 hours at 80°C and incubated with a [^{32}P]-labeled probe.

VI) Nick Translation of DNA Probe

Two hundred ng of DNA was placed in an 1.5 ml Eppendorf tube. Two μ l of the three non-radioactive nucleotide mixes (dTTP, dCTP, dGTP) was added to the tube and the contents were dried down. To the dry sample, 16 μ l H₂O, 2 μ l 10 X nick translation buffer, 2 μ l of 10X DNase (made from a stock solution of 1 mg/ml DNase with H₂O) and 2 μ l of α -[³²P]dATP was added. The mixture was incubated at 12°C for 30 minutes, then 1/4 μ l of DNA polymerase I was added and the incubation was continued at 12°C for one and half hours. 8 μ l of 0.25 M EDTA solution was added and the mixture was heated at 65°C for 10 minutes. The mixture was passed through a G-75 column to remove the excess [³²P]-dATP. The amount of the ³²P incorporated into the nick-translated samples was determined in a counter and 1-3 x 10⁶ cpm were used for the blots.

VII) Southern Blot Analysis

After running a regular agarose gel with DNA samples, the gel was placed in Southern blot solution I for 15 minutes at room temperature, and rinsed with water. The gel was then placed in Southern blot solution II for 45 minutes at room temperature and rinsed with water again. The gel was placed in Southern blot solution III for another 45 minutes.

A sponge was placed in a glass dish, the dish was filled with 10 X SSC, two pieces of the filter paper (Schleicher & Schuell) were placed on the sponge, then the gel was placed on top of them. A piece of nitrocellulose (the same size as the gel) was placed on the gel and air bubbles were smoothed out. Two sheets of filter paper were then placed on the top of the nitrocellulose, and a stack of paper towels was placed on top of the filter paper sheets. A weight was put on the top of the paper towels. The whole assembly was left for at least 4 hours and the nitrocellulose was then removed, air dried, and baked at 80°C for 2 hours.

VIII) Preparation of Plasmid DNA

The small scale plasmid preparation method of Birnboim and Doly (1979) was used. The large scale preparation method was according to Crosa and Falkow (1981).

3. Sequencing of plasmid DNA and Single-stranded DNA

Nucleotide sequencing of the clones was performed by the dideoxy method of Sanger *et al* (1977), using pUC18/19 and M13mp18/19 vectors and universal primers (Messing *et al.*, 1981).

4. Preparation of Anti-pilus Antisera

The method used was according to Watts *et al* (1982). One

hundred μg of pure PAK or PAO pili (in sterile 0.9% NaCl) and an equal volume of Freund's complete adjuvant were thoroughly mixed by passing the solution back and forth through a double hubbed needle attached to two syringes. This mixture was injected subscapularly and in the gluteal region of the rabbit. The same procedure was performed 2-4 weeks later except that Freund's incomplete adjuvant was used. The rabbit was bled from the ear vein 2 weeks later and sera were analysed for antibodies using the ELISA procedure described later in this section. Usually titers of $1/1-5 \times 10^6$ were obtained. If lower titers were obtained, a second injection of the protein in incomplete Freund's adjuvant was necessary. The rabbit was then bled at two week intervals until the desired quantity of sera was obtained.

5. SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE of proteins and peptides was carried out by the method of Lugtenberg *et al* (1975), unless specified otherwise. A slab gel apparatus with 0.75 mm Teflon spacers and 20 well comb, purchased from Bethesda Research Laboratories Inc., Gaithersburg, MD., was used. The running gels were 10-12 cm long and contained 15% acrylamide-0.27% methylene-bis-acrylamide, while the stacking gels were 1-2 cm long and contained 7% acrylamide-0.18% bis acrylamide.

1-5 μg purified protein samples were mixed with 20 μl of sample buffer consisting of 1.25 ml Tris.HCl (0.25 M, pH 6.8),

1 ml of 10% SDS, 1 ml of 50% glycerol, 0.2 ml of beta-mercaptoethanol, 0.2 ml of 0.04% (w/v) bromphenol blue and 0.35 ml of water. Samples were boiled for 20 seconds and loaded onto the gel. Electrophoresis was performed at 45 mA constant current until samples had travelled the desired distance. After electrophoresis, the gels were stained in protein gel stain solution for several hours, and then destained in protein destaining solution.

6. Whole Cell Protein Gel Sample Preparation and Membrane Purification

Whole cell protein gel samples were prepared as follows. Bacterial cells were grown to a density of 10^9 cells/ml. One ml culture was pelleted by centrifugation and the cells were resuspended in 100 μ l of SDS sample buffer (see above section). After boiling for 3 minutes and centrifuging the solution, 20 μ l of the supernatant solution was loaded onto the gel.

The preparation of *P. aeruginosa* strain cell membranes was carried out according to the procedure of Hancock and Nikaido (1978).

7. Immunoblot Procedure

The procedure used was based on the method of Towbin *et al* (1979). After electrophoresis, proteins were transferred to nitrocellulose paper using an Electro-blot Apparatus (E-C

Apparatus Corp., St. Petersburg, Fla.). Briefly, the gel was placed on a piece of filter paper, which was previously wetted with the transfer buffer, and placed on the cathode side of the electrophoresis grid. The gel was overlaid with a sheet of nitrocellulose which was pre-soaked with the buffer. All air bubbles between the gel and the nitrocellulose were removed by gently rubbing by hand, after which the entire assembly was covered with a second piece of wet filter paper. This "sandwich" was held in place on the grid with one layer of thin Scotch Brite Pads (3M Company), and the grid was closed and lowered into the electrophoresis tank containing enough transfer buffer to cover the grid. Electrophoresis was carried out for 2 hours at 4 mA/cm^2 , at which point proteins up to 68,000 daltons were completely transferred to the nitrocellulose as detected by Coomassie blue staining of the transferred gel.

After transfer, the nitrocellulose sheet was placed in 25-50 ml of Immunoblot buffer I containing an appropriate dilution of rabbit antisera (1:1000 dilution was used for PAK antiserum, and 1:300 was used for PAO antiserum in these studies) and incubated overnight at 37°C , with constant shaking. This was followed by a 2 hour washing step in the same volume of buffer I, at 37°C , with shaking. One μCi of ^{125}I Protein A in 50 ml of buffer I was added and incubated at 37°C for 2 hours with constant shaking. The nitrocellulose sheet was then briefly rinsed with buffer II and incubated with

buffer II for 1 hour at 37°C, with shaking. The nitrocellulose sheet was finally rinsed with distilled water, then dried prior to autoradiography using Kodak X-OMAT AR film. The film was exposed to the nitrocellulose sheet at -70°C for several days and developed using a Kodak X-OMAT automatic developer.

8. ELISA Assays

The principles of the enzyme-linked immunosorbant assay (ELISA), used for the detection of pilus specific antisera was described by Voller *et al* (1974). The assays used in this study were modified from the original assay. Microtiter plates (Dynatech, Immulon I plates, Alexandria, VA.) were coated with 200 μ l per well of a 5 μ g/ml solution of purified pili, in 0.5 M sodium bicarbonate coating buffer, overnight at 4°C. The antigen saturated wells were washed 3 X 3 minutes with PBS.T. buffer. After washing, the plates were incubated for 2 hours at room temperature with 200 μ l per well of the appropriate dilutions of antibody in PBS.T.BSA buffer. Unbound antibody was washed away with PBS.T and bound antibody-antigen complexes were detected with goat-anti-rabbit IgG conjugated to alkaline phosphatase (obtained from Boehringer Mannheim Corp., or Sigma and used at a 1:1000 dilution). After 2 hours incubation with the goat-anti-rabbit IgG solution, the plate was thoroughly washed with PBS.T and the alkaline phosphatase substrate, p-nitrophenyl phosphate (Sigma) was added to each

well as a 1 mg/ml solution in 10% (v/v) diethanolamine. After an appropriate time interval, the absorbance at 405 nm was determined with a Titertek Multiscan ELISA plate reader (Flow Laboratories Canada, Mississauga, Ont.). The titre or end point was described as the antibody dilution required to obtain an A_{405} of 0.05 after a predetermined length of time (about 15-30 minutes).

9. Electron Microscopy

A loopful of cells freshly grown on an L. Broth agar plate was suspended in 50 μ l of phosphate-buffered saline (pH 7.0), and 5 μ l of the suspension was placed for 30 seconds on a copper grid coated with a hydrophilic carbon film. After being washed two times with phosphate-buffered saline, the grid was stained with 0.5% sodium phosphotungstate and examined with a Philips EM420 electron microscope.

10. Cloning the GA Pilin Genes into the Broad Host Range Vector

The pilin genes of GA1 and GA2 are all within a 2.1-kilobase HindIII-PstI fragment. For expression of these pilin genes in different *P. aeruginosa* strains, it was necessary to clone them into a broad-host-range vector such as pKT210, which is 11.8-kilobases in length (Bagdasarian, *et al.*, 1981). The pilin genes were each cloned into the HindIII and PstI

sites of a pUC19 vector so that both the pilin gene (pil A) and the gene encoding β -lactamase (β -lac) were transcribed in the same direction (p. 88). These constructs were digested with EcoRI and ligated into the EcoRI site of pKT210 so that β -lac was proximal to the gene encoding chloramphenicol resistance. Chloramphenicol (Cm) and ampicillin (Ap) was used to select for the desired recombinants.

11. Plasmid Transfer by Conjugation

Conjugal plasmid transfer from E. coli to P. aeruginosa was performed using a procedure similar to that of Goldberg and Ohman (1984). Donor and recipient cells were incubated together with E. coli MM294 which contains the helper plasmid pRK2013. The helper plasmid has all the gene products necessary for plasmid conjugation with compatible plasmids. In a triparental mating, pRK2013 transfers itself into the donor cell and is then able to mobilize the donor's plasmid which has an origin of transfer but lacks the other transfer genes needed for conjugation (Willetts, 1984). The triparental mating was performed on a Millipore membrane filter (0.45 μ m pore size) incubated at 37°C on an L. Broth agar plate for 4 hours. The cells were then suspended in L. Broth medium and plated onto carbenicillin-Pseudomonas isolate agar plates to select for transconjugants.

12. Preparation of Bacteriophage

This method was based on that described by Pasloske (1989). Confluent lysis of bacterial growth on standard double-agar-layer plates at 37°C was used to obtain high titre phage stocks of PO4, B9 and f116. The phage were extracted with L. Broth for 3 hours, followed by centrifugation to remove debris. *P. aeruginosa* PA01-leu was the host bacteria for f116 and strain PAK was used for PO4 and B9. The titres of each of these bacteriophage preparations were 1×10^9 plaque forming units ml^{-1} .

13. The Spot Test for Bacteriophage Lytic Activity

This procedure was according to Pasloske (1989). The *P. aeruginosa* strains harboring the pilin plasmid constructs were grown at 30°C with shaking in the presence of carbenicillin to approximately 5×10^8 colony forming units ml^{-1} . Aliquots of 0.1 ml of each strain were pipetted into 2 ml of 0.7% soft agar, mixed well and poured onto carbenicillin-LB agar plates. Serial, ten-fold dilutions of each bacteriophage suspension were made in L. Broth and 2 μl of each dilution was pipetted onto the double-agar layer plate. The cells were incubated overnight at 30°C and local clearing indicated phage susceptibility.

Table II-1
Bacterial Strains, Plasmids and Bacteriophages

Strain	Relevant phenotype	Reference or source
Bacteria		
<i>Escherichia coli</i>		
JM83	$\Delta(lac-proAB)$, $lacZ\Delta M15$	Yanisch <i>et al</i>
MM294(pRK2013)	Mobilizing plasmid	Ruvkun and Ansel
DH5a	$hsdR17$ ($r_K^- m_K^+$) $lacZ$	Pasloske <i>et al</i>
<i>Pseudomonas aeruginosa</i>		
PAK	Auxotroph	ATCC 25102
PAO1	Prototroph	ATCC 25247
PAO-leu	<i>leu</i> mutant of PAO1	Paranchych
BLP2	$Trs01::pilA$ of PAK, Hg^r	Pasloske *
BLP3	$Trs01::pilA$ of PAO-leu, Hg^r	Pasloske *
Plasmids		
pUC18	MCS, Ap^r	Yanisch <i>et al</i>
pUC19	MCS, Ap^r	Yanisch <i>et al</i>
M13mp18	Sequencing vector with MCS	Yanisch <i>et al</i>
M13mp19	Sequencing vector with MCS	Yanisch <i>et al</i>
pKT210	Broad host range cloning vector	Bagdasarain <i>et al</i>
Bacteriophages		
PO4	Noncontractile tail, side binding	Bradley
f116	Noncontractile tail, side binding	Minimishima <i>et al</i>
Pf	Noncontractile tail, side binding	Pemberton
B9	Filamentous, tip binding	Hancock **

* Information adapted from B.L. Pasloske's Ph. D thesis, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7.

** Information adapted from R.E.W Hancock, Department of Microbiology, University of British Columbia, Vancouver, B.C., Canada V6T 1W5.

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CHAPTER III

MOLECULAR EPIDEMIOLOGY STUDIES ON THE PSEUDOMONAS
AERUGINOSA PILIN GENE*A. Introduction

Pseudomonas aeruginosa is a serious pathogen in patients with cystic fibrosis, thermal injury, and leukemia (Bodey et al., 1983). A common factor among these patients is an interruption of the natural barrier that protects them against invasion by bacteria. Although the specific cell surface alteration that allows the adherence of P. aeruginosa has not been well defined, there is increasing evidence that the adherence of P. aeruginosa organisms to the mucosal epithelial surface is mediated by filamentous adherence structures known as pili and that this adherence phenotype is necessary for bacterial colonization (Woods et al., 1980a; McEachran and Irvin, 1985; Paranchych et al., 1986; Wood et al., 1980b).

Studies of the epidemiology of P. aeruginosa in patients with CF have been severely hampered by the imprecision of existing methods for serotyping strains, based upon an

* A version of this chapter has been published. Brittan L. Pasloske, A. Mark Joffe, Qiming Sun, Kathy Volpel, William Paranchych, Fereshteh Eftekhari and David Speert. 1988. Infection and Immunity. 56:665-672

agglutination reaction between bacterial lipopolysacchride (LPS) and type-specific rabbit antisera (Fisher *et al.*, 1969). The serotyping scheme only works well for clinical isolates of *P. aeruginosa* which possess smooth LPSs (Pier and Thomas, 1982); however, most CF isolates of *Pseudomonas* are rough LPS strains, lacking the O-polysaccharide side chain which is responsible for the serotyping reaction (Hancock *et al.*, 1983). The majority of such strains are sensitive to the bactericidal effect of normal human serum and are either non-typable, auto-agglutinable or agglutinated by multiple typing sera (Hancock *et al.*, 1983; Hoiby and Ros  udal, 1980; Speert and Campbell, 1987; Speert *et al.*, 1982). Therefore, it seems likely that conventional serotyping methods do not offer the discriminatory power necessary to distinguish among isolates and provide answers to basic epidemiological questions.

The use of a DNA probe as an epidemiological marker for *P. aeruginosa* was first reported by Ogle *et al.* (Ogle *et al.*, 1987). Serial *P. aeruginosa* isolates from individual CF patients which varied in colonial morphology, serotype, and biotype were found to be identical when analyzed using a 741-bp probe of a *Pst*I-*Nru*I fragment derived from the upstream region of the exotoxin A gene.

A different DNA probe, a 1.2-kb *Hind*III fragment containing the PAK pilin gene (Pasloske *et al.*, 1985; Speert *et al.*, 1989), was used to study over 140 *P. aeruginosa* isolates from patients with CF, burns and cancer. Individual

PstI and HindIII chromosomal digestions of each isolate were performed, and the DNA was fractionated and then allowed to hybridize with this PAK pilin probe. Through these studies, 21 distinct restriction fragment patterns were found, whereas pilin gene cloning and sequencing studies only revealed 7 unique genes among the 17 cloned isolates so far (Paranchych et al., unpublished observations). Of the 140 isolates screened, many were serial isolates from individual patients, and in most instances, the restriction fragment pattern was of only one type despite apparent differences in O serotype, colonial morphology, and antibiotic sensitivity patterns. This gene probing method is also referred to as Restriction Fragment Length Polymorphism (RFLP) typing.

In this chapter, a summary of RFLP results of 32 P. aeruginosa isolates from CF patients and a further study of 5 serially collected P. aeruginosa isolates from a single patient will be presented.

B. RESULTS

1. Frequency of RFLP Types among Clinical Isolates of P. aeruginosa

A total of 32 clinical isolates of P. aeruginosa from six CF patients were studied using the RFLP typing method. All of them were from the patients in Vancouver, Canada. The characteristics and frequencies of the RFLP types, and LPS types for all the strains studied are summarized in Table III-1. Using two restriction enzymes (PstI and HindIII), five unique patterns were observed. In all six patients the RFLP pattern was the same in the sequential isolates indicating that the isolates were all related to a common ancestor.

2. Studies on Serial P. aeruginosa Isolates (CD Group) from a Cystic Fibrosis Patient

1) Phenotypic characteristics of the CD group

The colonial morphology, LPS serotyping of the five P. aeruginosa isolates from a single CF patient referred to as CD are given in Table III-1. The isolates were collected from the sputum of the patient in Vancouver on different dates.

2) Immunological properties of CD pilin

Whole-cell immunoblots of the five CD isolates were performed using either PAK or PAO antipilus serum. All of the isolates expressed CD pilin which cross-reacted

well with PAO antipilus serum, except CD3 pilin, which was not detected by either serum (Fig.III-1). The PAO antipilus serum exhibited weak cross-reactivity with PAK pilin, but there was no detectable cross-reactivity of PAK antipilus serum with PAO or CD pilin.

3) Analysis of genomic DNA by Southern hybridization with the PAK pilin gene probe

The chromosomal DNA from all five isolates was extracted and subjected to restriction enzyme digestion and hybridization with a 1.2-kb HindIII fragment containing the PAK pilin gene. The result, shown in Fig.III-2, indicated that all five CD isolates had identical RFLP patterns. This suggested close homology of the DNA in the vicinity of the pilin gene, and strongly suggested close relatedness of the pilin genes themselves.

4) Cloning and sequencing of the CD pilin genes

Cloning and sequencing experiments were performed by other individuals in the laboratory of Dr. Paranchych. The results are presented in chapter V for comparison purposes.

5) Extent of piliation of CD strains

Piliation of the CD strains was monitored by electron microscopy and bacterial sensitivity to the pilus-specific bacteriophage PO4. Pili were visualized by electron microscopy on strains CD2 and CD5 but not on

strains CD3, CD4, or CD10 (Table III-2). These observations were supported by bacteriophage PO4 sensitivity tests, which showed that strains CD2 and CD5 were sensitive to this pilus-specific phage, whereas strains CD3, CD4 and CD10 were phage resistant.

C. DISCUSSION

Strains of P. aeruginosa from CF patients are a unique class of bacteria, many of the isolates are mucoid and serum sensitive and possess rough O antigens in their LPSS. Conventional Pseudomonas typing systems are based on serological reactions with O antigens. However, because many Pseudomonas isolates are nontypeable, certain questions about the epidemiology of P. aeruginosa in patients with CF could not be answered. This has also frustrated attempts to characterize these strains for epidemiological purposes. With the new RFLP pilin gene typing system, it is possible to assign almost all isolates into an RFLP type. This provides a useful marker for epidemiological investigations, although the typing scheme appears to have limited discriminatory power. RFLP analysis using the exotoxin A probe somewhat provides greater discrimination among strains (Ogle et al., 1987), but it may not be able to type all P. aeruginosa isolates, since some strains lack the gene for exotoxin A (Vasil et al., 1986).

Five isolates of P. aeruginosa from a CF patient were examined with regard to various phenotypic and genotypic characteristics to determine whether the patient was colonized with one or several strains. The isolates were selected initially on the basis of observed differences in colonial morphology, serotype, and antibiotic resistance patterns,

which indicated that this patient may have been colonized with several unique Pseudomonas strains. However, southern blot analysis with a 1.2-kb HindIII DNA probe containing the PAK pilin gene suggested that all five strains were closely related genetically. The pilin genes of all five isolates were found to be identical. It thus appears that in some cases, CF patients are colonized with a single strain of P. aeruginosa, although examples of colonization with more than one strain were reported (Speert et al., 1989).

This study revealed that three of the CD isolates examined seemed to have acquired mutations which prevented normal expression of the pilus system. CD3 was a putative regulatory mutant which was unable to produce normal amounts of pilin, and CD4 and CD10 were putative assembly mutants which produced normal amounts of pilin but were unable to assemble the pilin subunits into intact pili. This suggested that after initial colonization, P. aeruginosa strains do not need to produce pili to continue colonizing the respiratory tracts of CF patients.

It is of interest that the CD strains yielded a PAK-like restriction fragment polymorphism, but the pilin turned out to be immunologically related to PAO pilin. Since strain PAO chromosomal DNA yields a different restriction fragment pattern, it was evident that the restriction fragment pattern, which depends on the locations of PstI and HindIII restriction sites in the flanking sequences of the pilin gene, need not

reflect the type of pilin gene actually present in the fragment. That the CD pilin gene is significantly different from that of strain PAK is evident in Fig.III-2, in which it can be seen that the 600-bp PstI fragment from the CD strains hybridized more weakly with the PAK pilin gene probe than did the same fragment from PAK chromosomal DNA. Interestingly, the sizes of the restriction fragments from the CD strains were virtually identical to those from the prototype PAK strain with the pilin gene probe, but the actual pilin gene was quite different from that of strain PAK, indicating that it is also useful to perform the serotyping method in addition to RFLP when identifying specific genotypes. It is important to emphasize the relative size of the restriction fragment when using RFLP method of analysis rather than the relative intensities of the bands.

The immunologic properties of the CD pilin proteins and the difference between CD pilin and the other known pilin proteins will be discussed in chapter V.

TABLE III-1 Characteristics of 32 *P. aeruginosa* isolates from patients with CF in Vancouver

Strain	Date	Colonial morphology	LPS serotypes	<u>Pst</u> I Fragment (kb)	<u>Hind</u> III Fragment (kb)	RFLP*
CD2	2-15-82	Mucoid	11	0.6, 1.5	1.2	3
CD3	2-15-82	Dwarf	AA	0.6, 1.5	1.2	3
CD4	2-15-82	Rough	AA	0.6, 1.5	1.2	3
CD5	2-24-83	Mucoid	11	0.6, 1.5	1.2	3
CD10	2-21-85	Classic	3	0.6, 1.5	1.2	3
LM4	7-10-86	Mucoid	3	4	1.2	5
LM5	7-10-86	Mucoid	3	4	1.2	5
LM6	7-10-86	Classic	3	4	1.2	5
LM7	7-10-86	Classic	3	4	1.2	5
LM8	7-10-86	Mucoid	3	4	1.2	5
LM9	7-10-86	Classic	3	4	1.2	5
TM1	7-10-86	Mucoid	6	5.5	2.5	4
TM2	7-10-86	Mucoid	6	5.5	2.5	4
TM3	7-10-86	Mucoid	6	5.5	2.5	4
TM4	7-10-86	Mucoid	6	5.5	2.5	4
TM5	7-10-86	Mucoid	6	5.5	2.5	4
TM6	7-10-86	Mucoid	6	5.5	2.5	4
NW3	4-8-83	Mucoid	PT	2.2, 3.7	>21	1
NW4	4-8-83	Classic	PT	2.2, 3.7	>21	1
NW5	7-15-85	Mucoid	PT	2.2, 3.7	>21	1
NW6	7-15-85	Classic	PT	2.2, 3.7	>21	1
DA1	7-11-87	Classic	6	2.2, 3.7	3	2
DA2	7-11-87	Rough	6	2.2, 3.7	3	2
DA3	7-11-87	Rough	6	2.2, 3.7	3	2
DA4	7-11-87	Mucoid	6	2.2, 3.7	3	2
DA5	7-11-87	Mucoid	6	2.2, 3.7	3	2
GA1	6-15-81	Dwarf	AA	2.2, 3.7	3	2
GA2	1-11-82	Mucoid	NA	2.2, 3.7	3	2
GA3	1-11-82	Dwarf	6	2.2, 3.7	3	2
GA5	7-14-83	Classic	NA	2.2, 3.7	3	2
GA7	3-27-84	Mucoid	6	2.2, 3.7	3	2
GA8	3-27-84	Classic	6	2.2, 3.7	3	2

* RFLP: Restriction Fragment Length Polymorphism. The numbers are the types of the RFLP from a total known group of 21.

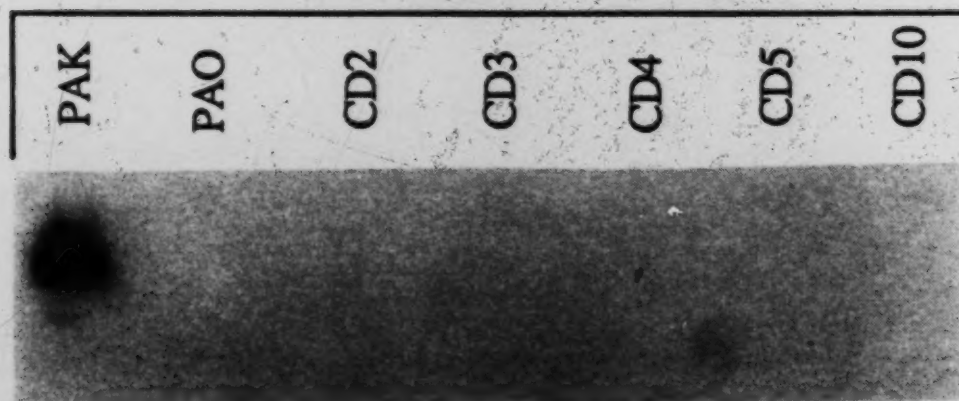
** PT, polytypeable; AA, autoagglutinable; NA, not agglutinated.

TABLE III-2 Pilus expression by *P. aeruginosa* isolates
(CD group) from a patient with CF

Strain	Pilin biosynthesis*	Presence of pili on cell surface	
		Electron microscopy	Phage P04 sensitivity
CD2	+	+	+
CD3	-	-	-
CD4	+	-	-
CD5	+	+	+
CD10	+	-	-

*Detected by whole-cell immunoblot analysis

PAK Specific Antiserum



PAO Specific Antiserum

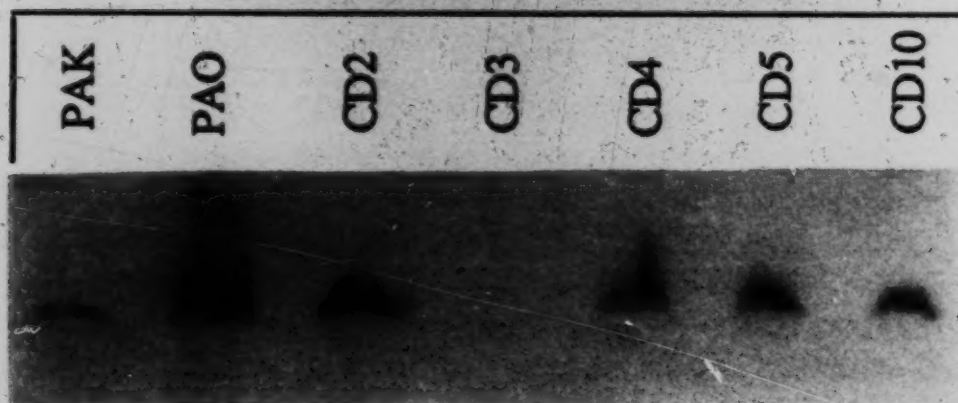


Fig.III-1 Autoradiograph of an SDS-PAGE immunoblot showing reactivity of pilin in whole-cell bacterial lysates from seven strains of *P. aeruginosa* with PAK and PAO antipilus polyclonal rabbit antisera. [^{125} I] protein A was used to indicate the positions of antibody-bound protein. The equivalent of 3×10^8 bacteria was applied to each lane for SDS-PAGE.

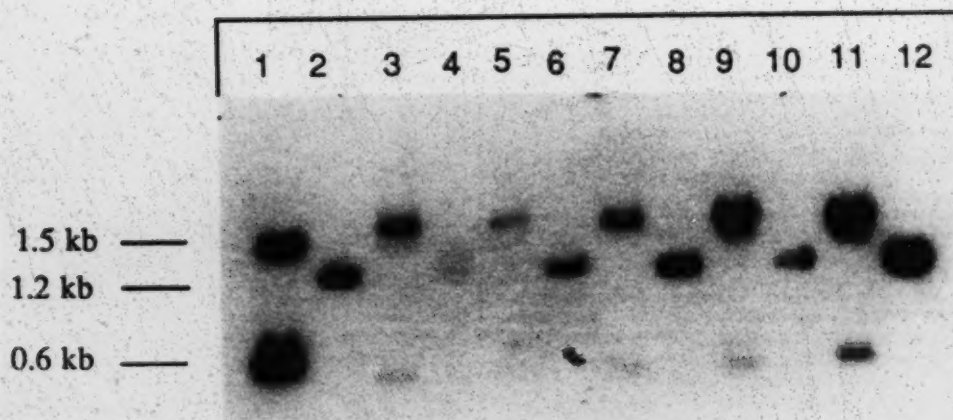


Fig.III-2 Restriction digests of *P. aeruginosa* chromosomal DNA probed with a nick-translated 1.2-kb *Hind*III fragment containing the *P. aeruginosa* PAK pilin gene. The genomic DNAs were digested with *Pst*I and *Hind*III and hybridized with a 1.2-kb PAK pilin gene probe. Odd-numbered lanes contained *Pst*I digests. Even-numbered lanes contained *Hind*III digests. Lanes: 1 and 2, PAK; 3 and 4, CD2; 5 and 6, CD3; 7 and 8, CD4; 9 and 10, CD5; 11 and 12, CD10.

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CHAPTER IV

MOLECULAR BIOLOGY STUDIES ON SERIAL CLINICAL ISOLATES OF
THE PSEUDOMONAS AERUGINOSA GA GROUPA. INTRODUCTION

The opportunistic pathogen, Pseudomonas aeruginosa, is a potential infectious agent in patients with leukaemia, cystic fibrosis or thermal damage (Bodey et al., 1983). These organisms produce chromosomally encoded, polar pili that are flexible, filamentous surface organelles with a diameter of 5.2 nm and an average length of 2500 nm (Weiss, 1971; Bradley, 1972; Bradley, 1980; Folkhard et al., 1981). The subunit monomer of a pilus is pilin (of molecular weight 15-18 kd), which is arranged in a helical array of five subunits per turn to form a pilus (Watts et al., 1983). Pilin proteins are also expressed by a variety of Gram-negative pathogens including Neisseria gonorrhoeae (Schoolnik et al., 1985), Moraxella bovis (Marrs et al., 1985), Moraxella nonliquefaciens (Froholm and Sletten, 1977), and Bacterioides nodosus (McKern et al., 1983). The pili produced by these bacteria belong to the N-methylphenylalanine (NMePhe) (Paranchych and Frost, 1988)

class of pili which have a unique NMePhe as their N-terminal residue.

In order to understand the molecular basis of biochemical, serological and antigenic diversity of Pseudomonas pili, serial clinical isolates were collected and immunoblot experiments were carried out using whole-cell preparations and PAK- or PAO-specific antiserum. The immunoblotting procedure showed that about 80% of the strains reacted with PAK antiserum, while many fewer strains reacted with PAO antiserum. Besides interstrain antigenic variations, the results also showed that there is some intrastrain heterogeneity among several groups of clinical isolates. The GA group was one such example of six closely related Pseudomonas isolates with interesting heterogeneity. The GA group consisted of a series of six P. aeruginosa isolates obtained from a single CF patient in Vancouver over a three year period. Some background information about this group has been listed in Table IV-1. All six isolates were studied by whole-cell immunoblotting experiments utilizing PAK- and PAO-specific antisera. Phage sensitivity assays were also carried out. Two out of six isolates from this group were further studied by pilin gene cloning, sequencing, and expression in mutant Pil strains PAK and PAO. The results of these experiments are presented and discussed in this chapter.

B. RESULTS

1. Immunological Properties of GA Pilin

Whole-cell bacterial lysates of the GA group of *P. aeruginosa* strains as well as strains PAK and PAO were subjected to SDS-PAGE (Fig.IV-1). Immunoblots of the six isolates were performed using either anti-PAK (Fig.IV-2) or anti-PAO (Fig.IV-3) pilus serum. Four out of six GA strains, GA1,3,7 and 8 reacted with anti-PAK serum at the positions indicating a molecular weight of about 17,000. All six GA strains reacted with anti-PAO serum and the positions of the pilin bands indicated different molecular weight values: GA1,3,7 and 8 pili were about 17,000, while GA2 and 4 pili were at 15,000. The different molecular weight values and immunological behavior of pilus encoded by these six GA strains have raised a question: what is the underlying basis of these interesting phenotypes?

2. Analysis of Genomic DNA by Southern Hybridization with the PAK or PAO pilin gene probes

When the chromosomal DNA preparations from isolates GA1 and GA2 were subjected to several restriction enzyme digestions and hybridized with a 1.2-kb *Hind*III fragment containing the PAK or the PAO pilin genes, nearly identical patterns were observed (Fig.IV-4, IV-5), indicating a high level of homology between the DNA from the two GA isolates.

This indicated a close relationship between the GA1 and GA2 pilin genes.

3. Cloning and Sequencing of the GA1 and GA2 Pilin Genes

Complete HindIII restriction enzyme digests of GA1 and GA2 chromosomal DNA were ligated into the pUC18 and pUC19 vectors and the resulting recombinant plasmids were screened using the 1.2-kb PAK pilin gene probe. For both GA1 and GA2, a 3-kb positive insert was purified and digested with PstI, and tested for hybridization with the PAK pilin gene probe. A 2.1-kb fragment was found to contain the pilin gene. Further digestions with other restriction enzymes revealed that the 2.1 kb fragment contained several Sau3A sites and single SphI and HincII sites. HindIII-SphI, SphI-HincII, HincII-PstI, Sau3A-Sau3A fragments were subcloned into sequencing vectors M13mp18 and M13mp19. Sequencing of GA1 and GA2 pilin genes and the flanking regions was performed by using the dideoxy method (Sanger et al, 1977) with the universal and synthesized internal primers. The restriction mapping of the 3-kb HindIII fragment of GA1 and GA2 and the sequencing strategy are shown on Fig.IV-6. The sequences of GA1 and GA2 HindIII-PstI fragments were found identical and are listed in Fig.IV-7.

4. Expression of Cloned Pilin Gene in *P. aeruginosa* cells

To gain some indication as to why apparently identical pilin genes give rise to different pilin phenotypes when examined by immunoblotting, the effect of host cell type on pilin gene expression was examined. Two sets of host cells were chosen: i) mutant PAK and PAO Pil⁻ strains BLP2 and BLP3; ii) Wild type strains GA1 and GA2. Also, three kinds of clones were used for the experiments: i) a clone containing PAK wild type pilin gene; ii) a clone containing another *P. aeruginosa* strain P1 pilin gene (Pasloske *et al*, 1988). DNA hybridization using a P1 pilin gene probe strongly reacted with GA1 and GA2 pilin gene DNA. P1 pilin has a higher molecular weight value than the classical PAK and PAO pilin (Pasloske *et al*, 1988); iii) clones containing GA1 or GA2 wild type pilin genes.

pUC-19 chimeras containing these different pilin genes were first digested with *EcoR* I and ligated into the *EcoR* I site of a broad host range vector pKT210 (see chapter II for more details) (Fig.IV-8). The constructs were transformed into *E. coli* strain DH5 α , then transferred to different host cells by the means of triparental mating with the helper plasmid MM294.

1) Expression of the GA1 and GA2 pilin genes in PAK and PAO Pil⁻ strains.

The expression of the GA1 or GA2 pilin gene in PAK pil⁻ strain BLP2 and PAO Pil⁻ strain BLP3 was examined by

immunoblotting with PAK- and PAO-specific antisera. The results of the experiment are shown in Figs. IV-9 and IV-10. The experiments revealed that the GA1 and GA2 pilins expressed in BLP2 and BLP3 reacted more strongly with anti-PAO serum, and the molecular weight values appeared to be 15,000. This suggested that there may be a difference in the expression of GA1/GA2 in BLP2/BLP3 versus their expression in the wild type strains GA1/GA2 because of differences in pilin processing.

2) Expression of *P. aeruginosa* P1 and PAK pilin genes in GA1 and GA2 host strains

Two wild type pilin genes, with different pilin protein sizes, were examined with respect to their expression in GA1 and GA2 wild type strains. As mentioned earlier, the molecular weight of PAK pilin is 15,000 while that of P1 pilin is 17,000. The results of the experiment are shown on Figs. IV-11 and IV-12. Immunoblotting showed that both P1 and PAK pilin genes were expressed in GA1 and GA2 host cells. However, it seems that the expression of these foreign chimeras might inhibit the expression of the GA1 and GA2 pilin proteins. In the case of GA1, it was difficult to establish whether the single band shown in the "GA1-200" lanes of Figs. IV-11 and IV-12 was P1 pilin only or both P1 and GA1, since the pilin bands of these two strains were similar. However, the "GA1-500" lanes only showed one band with a

molecular weight value of 15,000. It is possible, therefore, that GA1 pilin expression was inhibited by the expression of the PAK pilin gene. Because GA2 pilin cannot be detected by PAK antiserum, an experiment using only PAO antiserum was carried out to reveal pilin expression. In Fig.IV-12, only one band of molecular weight 17,000 was seen in the GA2-200 lane, suggesting that GA2 pilin expression was inhibited by P1 pilin gene expression. As in the comparison of GA1 and "GA1-200" with anti-PAO antiserum (Fig.IV-12), the comparison of GA2 and "GA2-500" was difficult because both GA2 pilin as observed with anti-PAO antiserum, and PAK pilin have apparent molecular weight of 15,000 (Fig.IV- 12).

5. Bacteriophage Sensitivity Assays

Further studies directed at finding the differences in GA1 and GA2 pilin expression and assembly were carried out with bacteriophage sensitivity assays. The *P. aeruginosa* strains harboring different recombinant plasmids containing different pilin genes were exposed to four kinds of bacteriophages. The sensitivities of these strains to these phages are listed in Table IV-2. The results indicated that the cloned P1 and PAK pilin genes were expressed and pili were assembled within these different host strains. Different sensitivity patterns were observed among these host strains containing various pilin-containing chimeras.

GA1 and GA2 wild type strains are sensitive (GA1 is

slightly more sensitive) to phages PO4, B9 and f116, and completely resistant to phage Pf. Expression of the P1 pilin gene resulted in conversion to PO4 phage resistance and greater sensitivity to f116. Expression of the PAK pilin gene in GA1 and GA2 did not change the sensitivity patterns of these two strains significantly. This is particularly interesting because *P. aeruginosa* PAK are normally sensitive to the filamentous Pf phage. The observation that GA1-500 and GA2-500 were Pf-resistant suggested that PAK pilin assembly may be different in these strains than in the wild-type PAK strain.

Mutants of PAK and PAO Pil⁻ are resistant to all these four phages. Expression of the GA1 and GA2 pilin genes in these strains resulted in sensitivity to PO4, B9 and f116, and resistance to Pf phage. This pattern is similar that observed with GA1 and GA2 wild type strains. There is a significant difference in the expression of GA1/GA2 pilin genes in the PAK Pil⁻ strain and the PAO Pil⁻ strain. Expression and assembly levels appeared to be much higher in the PAO Pil⁻ strain than in the PAK Pil⁻ strain, since PAO Pil⁻ strains containing GA1 or GA2 expression chimeras were much more sensitive to PO4, B9 and f116 than PAK pil⁻ strain containing GA1 or GA2 expression chimeras.

C. DISCUSSION

1. Possible Causes of Different Immunoblotting Patterns between GA1 and GA2

P. aeruginosa strains are able to change many surface characteristics to adapt to different environmental conditions. These modifications evidently do not involve extensive chromosomal mutations but are due almost entirely to phenotypic alternations in the expression of surface macromolecules such as LPS, alginate, or outer membrane proteins (Brown and Williams, 1985). These changes lead to altered colonial morphology, serotypes, phage and pyocin susceptibility patterns and antibiograms (Brown and Williams, 1985; Ogle et al., 1987). These differences in phenotypic properties were usually construed as evidence for unique strains of P. aeruginosa (Hoiby and Rosenthal, 1980; Komiyama et al., 1987). However, more recent investigations have shown that these phenotypic traits may provide misleading information with regard to epidemiological typing of Pseudomonas strains, whereas the use of DNA probes for specific regions of the P. aeruginosa chromosome appear to provide a more reliable approach to strain typing.

When six clinical isolates from one patient (GA) were examined by whole-cell immunoblotting experiments using PAK and PAO antisera, unique intrastrain antigenic variation

patterns differing in molecular weight were observed: a 17-kd band was detected with PAK antiserum in strains GA1, GA3, GA7 and GA8. When PAO antiserum was used, 17-kd bands were detected in the same strains (i.e., GA1, GA3, GA7 and GA8) and 15-kd bands were detected in strains GA2 and GA4. Two out of six isolates, GA1 and GA2 were further studied by southern DNA hybridization, restriction enzyme mapping, cloning, nucleotide sequencing, and expression of the pilin genes in different *P. aeruginosa* host strains. Piliation of the GA group strains was monitored by testing for bacterial sensitivity to pilus-specific bacteriophages. None of these approaches revealed any significant differences between the phenotype and genotype of the cloned pilin genes of GA1 and GA2. The nucleotide sequences of the cloned GA1 and GA2 pilin genes and their flanking regions were found to be identical. These observations revealed an interesting anomaly: what is the cause for the intrastrain immunoblot patterns that were observed with the wild-type GA1 and GA2 strains? There are several possible explanations: 1) One obvious possibility is that the GA1 and GA2 strains were mixed prior to the molecular biology studies and that only one strain was cloned and sequenced twice. 2) The second possibility is that other cryptic factors are involved in producing these interesting immunoblotting patterns.

To address possibility 1), it should be mentioned that only original bacterial stocks were used to extract

chromosomal DNA for the cloning and sequencing studies. Repetitive cloning and sequencing of each isolate was carried out with extra care to provide a higher level of confidence regarding the source of samples. The most likely explanation points to one or two unknown factor(s) in strains GA1 and GA2 which cause the GA2 pilins to associate differently than GA1. The difference(s) could be due to a change in the protein conformation (which could change a protein's antigenicity), or a change in the charge distribution of the protein, which would change the mobility of the protein during electrophoresis. This has been observed. For example, single amino acid changes in the PAK pilin gene, which result in charge differences, sometimes give rise to significant changes in mobility in SDS polyacrylamide gel electrophoresis (Pasloske and Paranchych, unpublished results). Further studies are required to identify the responsible factor(s).

2. Analysis of the GA Pilin Gene and Its Flanking Regions Sequence

Although the pilin genes of GA1 and GA2 are within a 0.8-kb SphI-HincII fragment, the whole 2.1-kb HindIII-PstI fragment was sequenced and revealed significant sequence information both upstream and downstream of the pilin gene.

As mentioned in Chapter I, pilin gene expression from P. aeruginosa strains was found to be controlled by NtrA-dependent promoters. NtrA, the gene product of ntrA, is an

alternative, 54-kd sigma factor initially discovered to be involved in the transcription of the genes which fix and assimilate nitrogen in microorganisms (Gussin *et al.*, 1986). The GA pilin gene also appears to be driven by an NtrA-type promoter recognized by Johnson *et al.* (1986). The putative NtrA-dependent promoter is a region 10 to 26 base pairs upstream of the transcription start site, where the *ntrA* gene product, acting as a sigma factor, is believed to be required for transcription initiation. In many enteric bacteria, genes involved in nitrogen assimilation, such as *glnA*, as well as the nitrogen fixation genes in organisms like *Klebsiella pneumoniae*, have NtrA-dependent promoters, and these genes are regulated by the gene products encoded by *ntrC* and *nifA*, respectively (Gussin *et al.*, 1986). NtrC and NifA both recognize specific sequences upstream of the genes that they positively regulate. Analysis of the DNA upstream of the pilin genes from *P. aeruginosa* PAK (Sastry *et al.*, 1985), PAO (Sastry *et al.*, 1985), PA103 (Johnson *et al.*, 1986), P1 and K122-4 (Pasloske *et al.*, 1988), GA1 and GA2 revealed a conserved sequence among all of these strains. This sequence had a strong homology with the NifA recognition sites of the nitrogen fixation genes which are normally located 100 to 150 nucleotides upstream of the transcription initiation site. Table IV-3 outlines the hypothetical NifA recognition sites associated with each pilin gene. These sequences are identical to the NifA-binding site consensus sequence of TGT-N₁₀-ACA

(Buck et al., 1986) except for the difference in the spacing between the conserved trinucleotides, so that N is 11 instead of 10. Since *P. aeruginosa* is not known to have a *nifA* gene, an *ntrC*-type regulatory gene may be responsible for this regulation. Pilin expression and nitrogen assimilation (Janssen et al., 1981) in *P. aeruginosa* may both be regulated by an NtrC-like protein which associates with the NifA-like recognition site, since the *ntrC* and *nifA* genes are often interchangeable for gene activation (Gussin et al., 1986).

Regions of possible importance in the 3'-flanking sequence have also been observed. There are three inverted repeats from nucleotides 1160 to 1183, 1333 to 1602 and 1886 to 2077 (Fig.IV-7). These sequences could form stem and loop structures and may be responsible for the termination of mRNA transcription (Rosenburg and Court, 1979) or mRNA stability (Stern et al., 1984). The inverted repeats were also found in the 3'-flanking sequence of the pilin genes of PAK and PAO (Sastry et al., 1985).

The protein sequence of GA pilin was also compared with the other known pilin sequences, and the significant results will be presented in Chapter V.

TABLE IV-1 Characteristics of *P. aeruginosa* isolates
from a patient with CF (GA group)

Strain ^a	Isolate date	Colony morphology	LPS serotype ^b	Pilin serotype ^c
GA1	1-15-81	Dwarf	AA	PAK, PAO
GA2	1-11-82	Mucoid	N/A	PAO
GA3	1-11-82	Dwarf	6	PAK, PAO
GA5	7-14-83	Classic	N/A	PAO
GA7	3-27-84	Mucoid	6	PAK, PAO
GA8	3-27-84	Classic	6	PAK, PAO

^aAll the GA group strains were isolated from the sputum of a CF patient in Vancouver, B.C., Canada.

^bLPS serotyping was done with the International Antigenic Typing System (Difco Laboratories, Detroit, Mich.).

^cDetermined by immunoblotting with polyclonal antiserum. N/A, Non-agglutinated.

TABLE IV-2 Bacteriophage Sensitivity Assay

Pili expressed	Lowest titre producing confluent lysis ^a			
	PO4	B9	Pf	f116
PAK (wild type)	4	6	5	7
PAO (wild type)	7	7	R	5
GA1 (wild type)	6	8	R	6
GA1-200 (P1 clone)	R	8	R	8
GA1-500 (PAK clone)	5	7	R	7
GA2 (wild type)	7	8	R	7
GA2-200 (P1 clone)	R	8	R	7
GA2-500 (PAK clone)	6	7	R	7
PAK (<u>pil</u>)	R	R	R	R
PAO (<u>pil</u>)	R	R	R	R
PAK <u>pil</u> -GA1/2	8	9	R	9
PAO <u>pil</u> -GA1/2	3	6	R	4

^aThe numbers indicate the $\log_{10}$ of the lowest phage titre producing confluent lysis. The original concentrated titres of each of these bacteriophage preparations was 1×10^7 plaque forming units ml^{-1} . Ten-fold dilutions were made to determine the lowest titre producing confluent lysis.

TABLE IV-3 Putative NifA recognition sites from the pilin genes of several *P. aeruginosa* isolates

Strain ^a	Sequence ^b
Consensus	TGT.....ACA
PAK	GGAGTCCGGCTGTCAAAAAGTGTACATCCTGTCCGT
PAO	GAAATCCAGCTGTCAAAAATGTACATCCTGTCCGT
PA103	GAAATCCAGCTGTCAAAAATGTACATCCTGTCCGT
K122-4	GAAATCCAGCTGTCAAAAATGTACATCCTGTCCGT
P1	GATTCCGACATG <u>CC</u> AAAAAGTGTACATATTG <u>CCGGC</u>
GA1/2	GATTCCGAGATG <u>CC</u> AAAAAGTGTACATATTG <u>CCGGC</u>

^a For the strains other than GA1/2, see the reference Pasloske et al., 1988.

^b Underlined nucleotides indicate differences from the predominant nucleotide of that position.

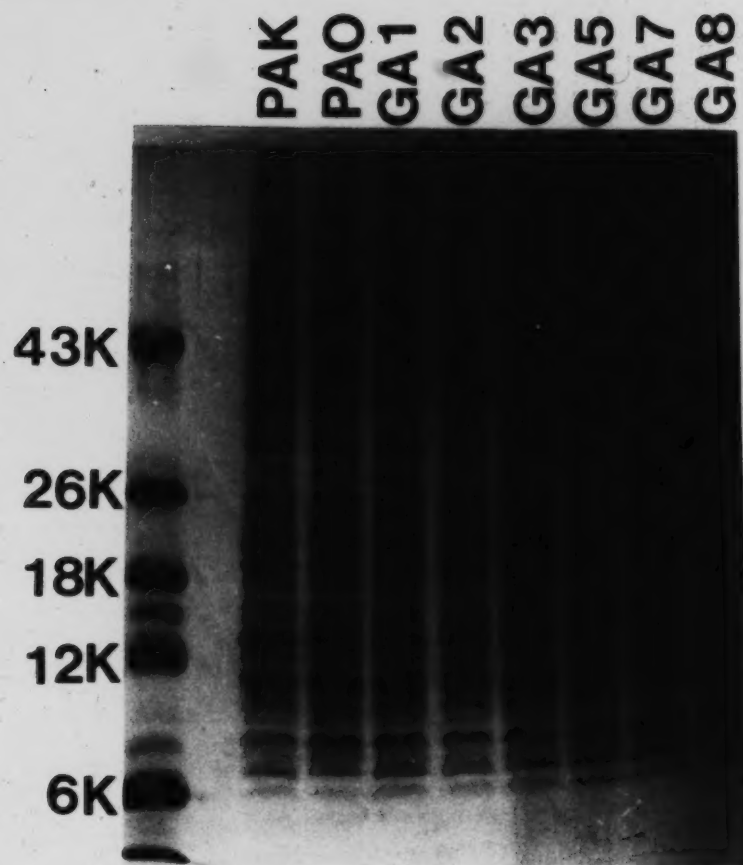


Fig.IV-1 Coomassie-Blue-stained gel of whole-cell bacterial lysates from eight strains of *P. aeruginosa*. The equivalent of 3×10^7 bacteria was applied to each lane.

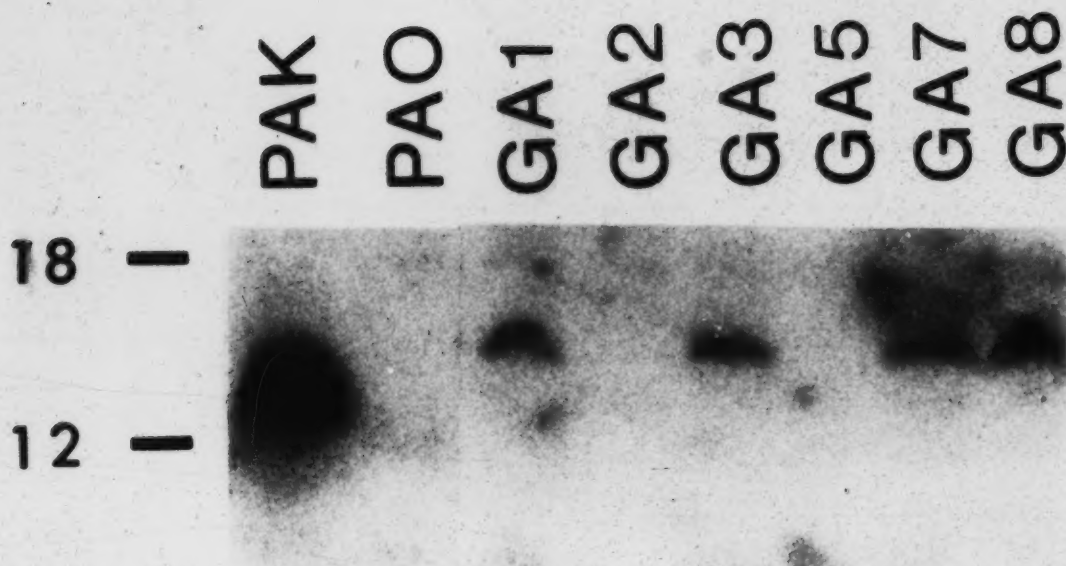


Fig.IV-2 Autoradiograph of an SDS-PAGE immunoblot showing reactivity of pilin in whole-cell bacterial lysates from eight strains of *P. aeruginosa* with anti-PAK pili polyclonal rabbit antiserum. [^{125} I] protein A was used to indicate the positions of antibody-bound protein. The equivalent of 3×10^7 bacteria was applied to each lane for SDS-PAGE.

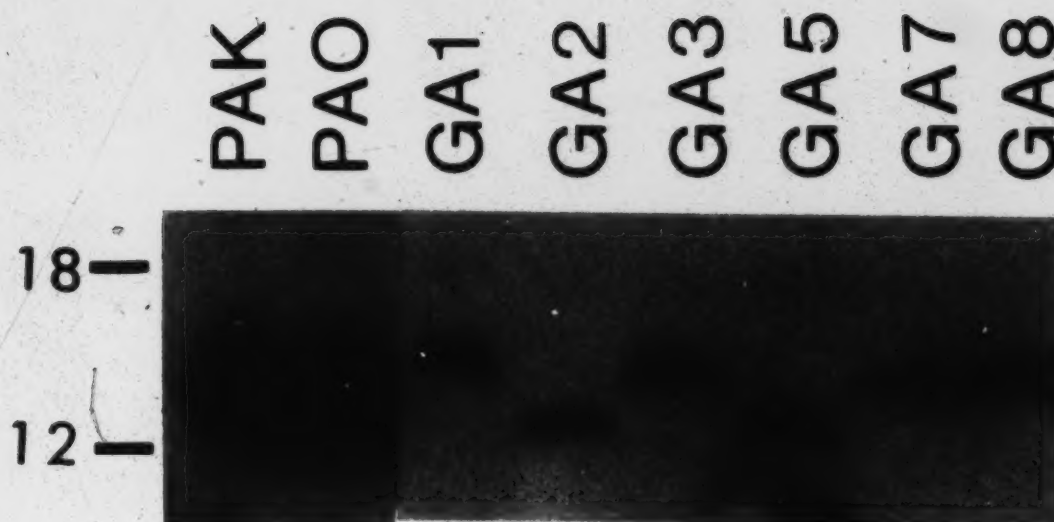


Fig.IV-3 Autoradiograph of an SDS-PAGE immunoblot showing reactivity of pilin in whole-cell bacterial lysates from eight strains of *P. aeruginosa* with anti-PAO pili polyclonal rabbit antiserum. [^{125} I] protein A was used to indicate the positions of antibody-bound protein. The equivalent of 3×10^7 bacteria was applied to each lane for SDS-PAGE.

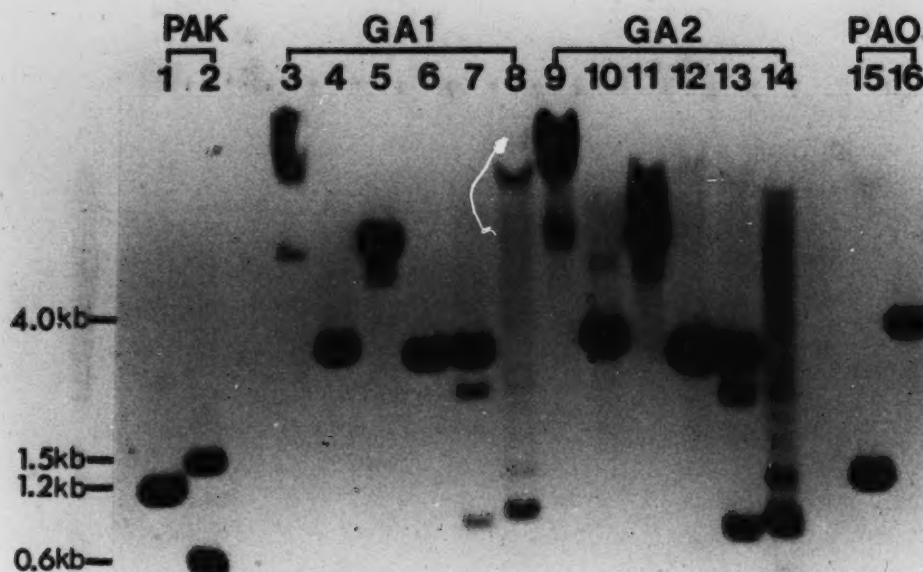


Fig.IV-4 Restriction enzyme digests of *P. aeruginosa* chromosomal DNA probed with a nick-translated 1.2 kb *Hind*III fragment containing the *P. aeruginosa* PAK pilin gene. The genomic DNAs were digested with various enzymes. Lanes: 1, *Hind*III; 2, *Pst*I; 3, *Bam*HI; 4, *Cla*I; 5, *Eco*RI; 6, *Hind*III; 7, *Pst*I and *Hind*III; 8, *Sau*3A; 9, *Bam*HI; 10, *Cla*I; 11, *Eco*RI; 12, *Hind*III; 13, *Pst*I and *Hind*III; 14, *Sau*3A; 15, *Hind*III; 16, *Pst*I.

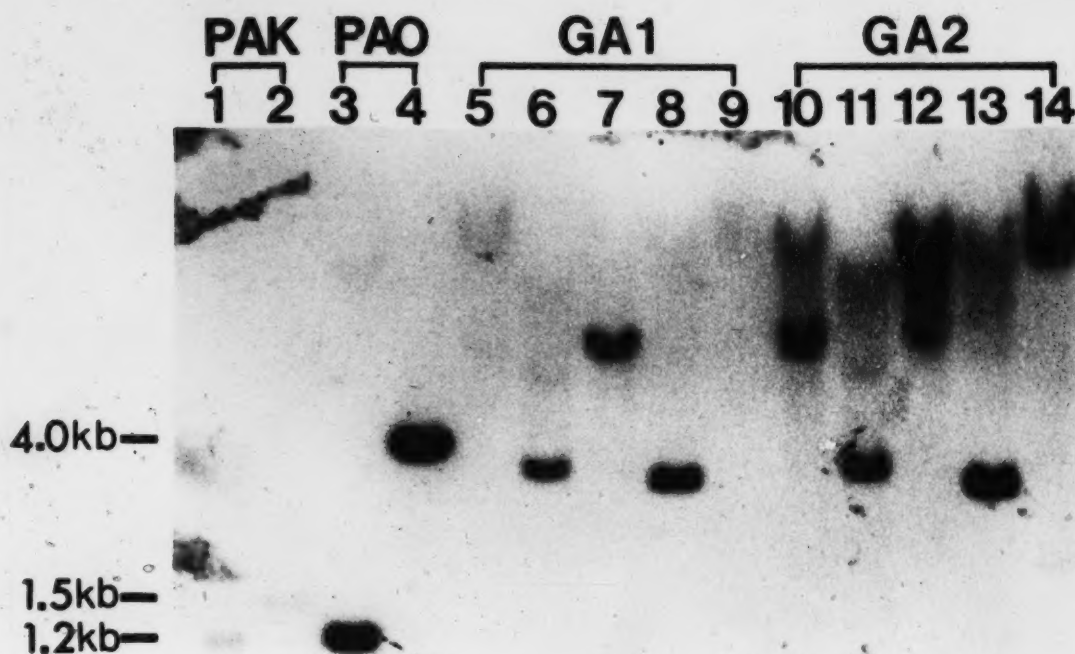


Fig.IV-5 Restriction enzyme digests of *P.aeruginosa* chromosomal DNA probed with a nick-translated 1.2-kb *Hind*III fragment containing the *P. aeruginosa* PAO pilin gene. The genomic DNAs were digested with various enzymes. Lanes: 1, *Hind*III; 2, *Pst*I; 3, *Hind*III; 4, *Pst*I; 5, *Bam*HI; 6, *Cla*I; 7, *Eco*RI; 8, *Hind*III and *Pst*I; 9, *Pst*I; 10, *Bam*HI; 11, *Cla*I; 12, *Eco*RI; 13, *Hind*III and *Pst*I; 14, *Pst*I.

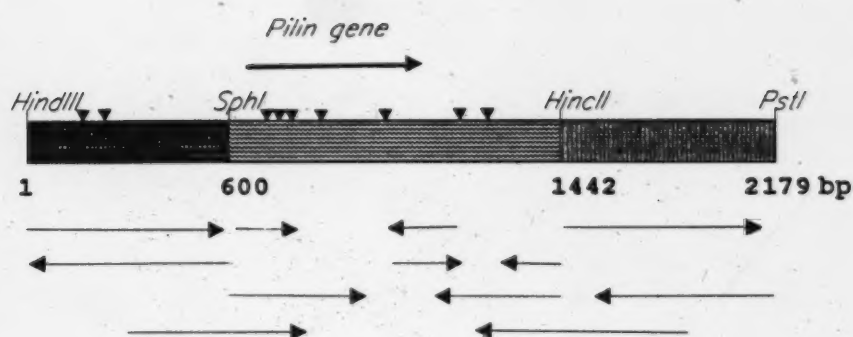


Fig.IV-6 Restriction enzyme map of a HindIII-PstI fragment containing the pilin gene of GA1/GA2 and its flanking regions. "▼" indicates the Sau3A sites. Bold arrows indicate the location of pilin gene and direction of transcription. The direction of the sequenced DNA fragments is shown by arrows beneath the restriction map.

20 40 60
AAGCTTCTCGATGGCTACGCCGAGCTTGTCTGTCCTCTACCAGCAGGGCTTCGGTGGTGAG
 80 100 120
GCCGGTGCTGAACTGGATATCGGTGACCGCCTGATGGTTGGTCGGGTCGGAGAGACCGAT
 140 160 180
GAACAGTTTGTGCGCGTTTCCACAGCGGCAACACGCGGTGCTGGCGGACCAGGCGCTC
 200 220 240
GCTGATGACATTCCCTTCGGAATGCTTTCCCGATCCAGGCTGTTTCAGGTCGCAATAGGCG
 260 280 300
ATGCGAAACTGCTCGGCGGCCACCTCGACCAACGCGCGGCTCTGCACCAGCTTGGCCTGG
 320 340 360
ACCAGGTGCTGACCCAGCGACTGCTTGTTGCGCGCGGCCTGCACCTGGGCTGGGCGGGCG
 380 400 420
GTCTTTTCGTGAGGAGTTCGCTTAGGACCAGTTGCTTGGACAGGCCGCTGAGAGGCGTG
 440 460 480
GAATCGTTCATTGGGGAACATCTGAAGAAGGCTTGAGCGAGACTTATAACGCAGATGCCA
 500 520 540
AAAGGCTGCCAAACCGACGATTCCGAGATGCCAAAAAGTGTCACATATTGCCGGCAGGGG
 560 580 600
AGAGCGGAACACTTTCGAAGTCTCCGCGAGAGTTGCGAAAGGAGGGCGTTTGAAAGGTTG
 620 640 660
GCATGCAACCTGCTTTGGAGGGGGAAGGCGTTTGGCCTATACATACCACATGGAGATATT
 680 700 720
CATGAAAGCTCAGAAGGGTTTTACTCTGATCGAACTGATGATCGTGGTCGCGATCATCGG
MetLysAlaGlnLysGlyPheThrLeuIleGluLeuMetIleValValAlaIleIleG
 740 760 780
CATCCTGGCCGCCATTGCCATCCCGCAATACCAGGACTACACCGCCCGTACCCAGGTGAC
yIleLeuAlaAlaIleAlaIleProGlnTyrGlnAspTyrThrAlaArgThrGlnValTh

(continued on next page)

Fig.IV-7 Nucleotide sequence of a 2179-bp HindIII-PstI region containing the pilin genes of *P. aeruginosa* isolates GA1 and GA2. Bold-printed sequences show several restriction enzyme sites. Bold and double-underlined regions indicate the putative NifA recognition site. Bold and single-underlined regions are the consensus sequences for the putative NtrA-dependent promoters. Inverted repeats are indicated by the arrows.

800 820 840
 CCGTGCCGTGAGTGAAATCAGTGCCTGAAGACCGCTGCGGAGTCGGCGATTCTGGAAGG
 rArgAlaValSerGluIleSerAlaLeuLysThrAlaAlaGluSerAlaIleLeuGluGl
 860 880 900
 CAAGAAGCTCGTTTCCAGCGATACCCCGGAAACAATGAATATGATCTTGGCTTTACCAG
 yLysLysLeuValSerSerAspThrProGlyAsnAsnGluTyrAspLeuGlyPheThrSe
 920 940 960
 CTCTACTCTGCTTACTGGTAGCGGTAAGGGCCAGATCAAGATTGACAAAGCTGATACCGC
 rSerThrLeuLeuThrGlySerGlyLysGlyGlnIleLysIleAspLysAlaAspThrAl
 980 1000 1020
 AACTCCGGAGATTTCTGGTACTCTGGGCAACTCTTCTGGTAAGGGTATCGCTGGCGCTGT
 aThrProGluIleSerGlyThrLeuGlyAsnSerSerGlyLysGlyIleAlaGlyAlaVa
 1040 1060 1080
 CATCACTGTCAAGCGTGATGATAAAGGAGTATGGACCTGCGGCATCACTGGTTCGCCGAC
 lIleThrValLysArgAspAspLysGlyValTrpThrCysGlyIleThrGlySerProTh
 1100 1120 1140
 CAACTGGAAAACCAACTACGCCCGGCTAACTGCCCGAAATCCTAATAGGTTTTTCGGTA
 rAspTrpLysThrAsnTyrAlaProAlaAsnCysProLysSer
 1160 1180 1200
 TGGTCTAGTGGTAAAAGAGAAAACCCCGCATTGCGGGGTTTTTTCATTACGGACTCGCGA
 1220 1240 1260
 TGAATCGAACAAGAGTGATCTGGCAAGGCGGGATTGCTTGCCTTCTTTCGTAGGGCTTG
 1280 1300 1320
 CACTGCTATCCCTGATAGGCAGCTATGCTGCTTTGTTTCGACTGTAATTCATTATGGGGTT
 1340 1360 1380
 TCTCCGAAGTGGCAGCAGCAGCGCATATTCCAGTTGCTGTTGCTGAGTGGCTCGTCA
 1400 1420 1440
 CTGTTTTTTTTCTTGTATTTTCCTTCTTCGTTTATTAGGCGAAAGGTTTTTGCCGCTCTA
 1460 1480 1500
 TTGTTGACTCTCGGGCTGGGTGTTTTTTCCTCCTTTCTTTCTGATAATTTTTCCTGGGCG
 1520 1540 1560
 CTCAAGGAGTGGTCGGTATTCGCCGGCCTGATGCTGTTCTCCTTCAATATTTCCGTCAGT
 1580 1600 1620
 CCGGAGTGGGTTCCGCGTGCCGCTATCTGGGGGCTGGTCTGTTGGGAGGGTTCTTCTGC

(continued on next page)

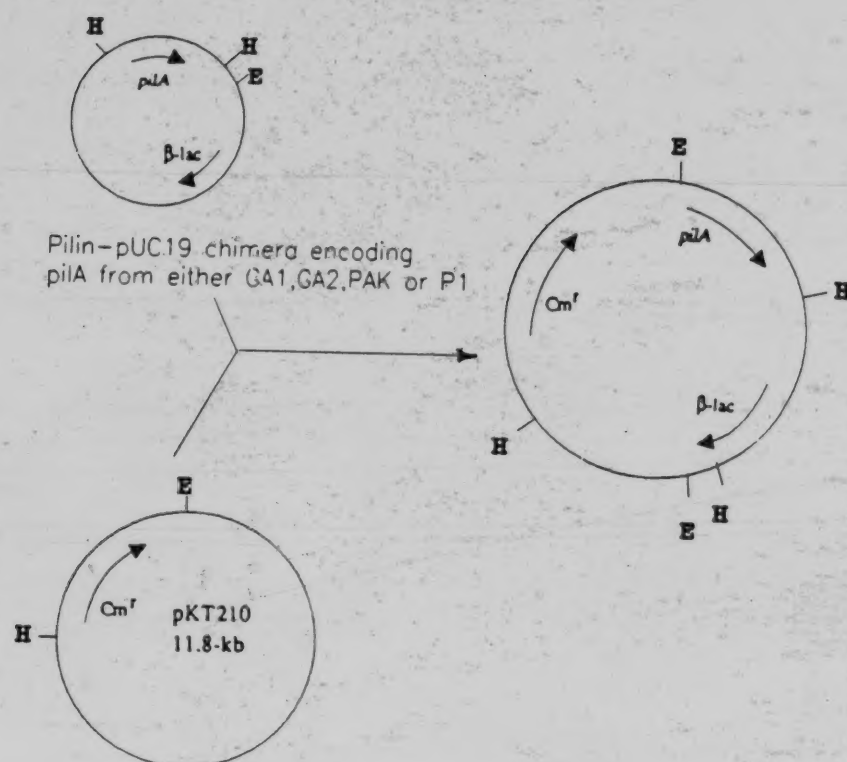


Fig.IV-8 Cloning of the pilin gene-containing chimera into the broad host-range vector pKT210.

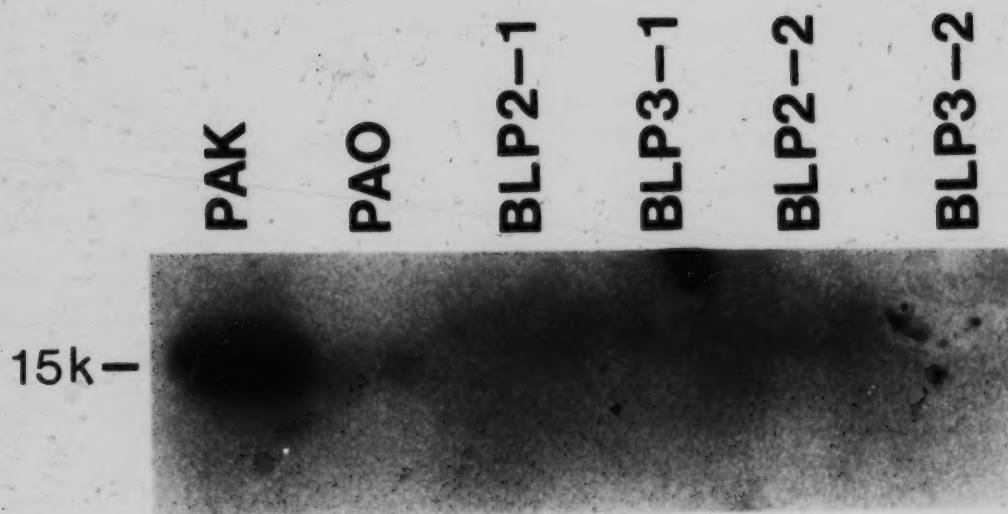


Fig.IV-9 Autoradiograph of an SDS-PAGE immunoblot showing reactivity of pilins expressed in whole-cell bacterial lysates of PAK and PAO pil mutants BLP-2 or BLP-3 containing recombinant plasmids containing GA₁ or GA₂ pilin genes. Anti-PAK polyclonal rabbit serum and [¹²⁵I] protein A were used to indicate the expression of antibody-bound protein.

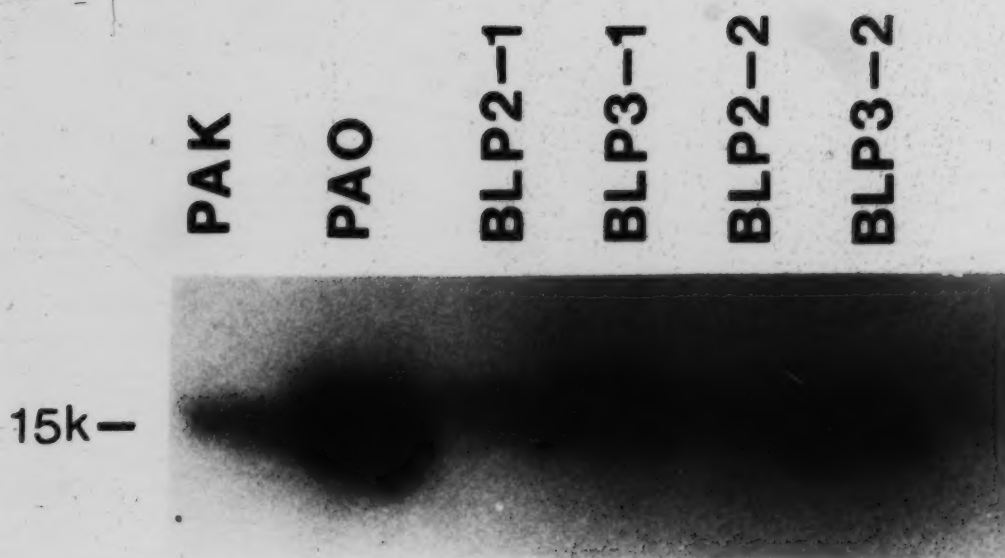


Fig.IV-10 Autoradiograph of an SDS-PAGE immunoblot showing reactivity of pilins expressed in whole-cell bacterial lysates of PAK and PAO pil mutants BLP-2 or BLP-3 containing recombinant plasmids containing GA₁ or GA₂ pilin genes. Anti-PAO polyclonal rabbit serum and [¹²⁵I] protein A were used to indicate the expression of antibody-bound protein.

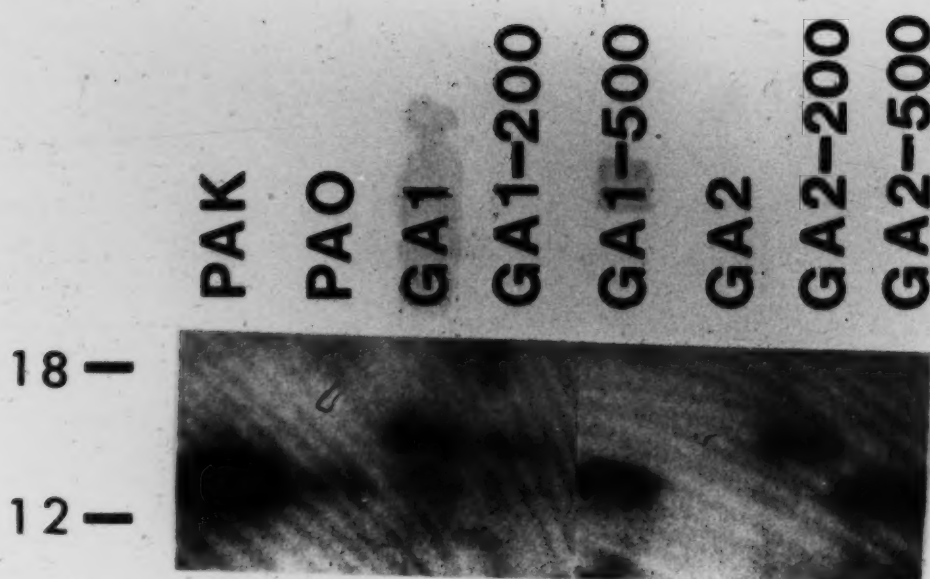


Fig.IV-11 Autoradiograph of an SDS-PAGE immunoblot showing reactivity of pilin expression in whole-cell bacterial lysates from eight strains of *P. aeruginosa*, PAK, PAO, GA1, GA2, GA1 or GA2 containing pBP200 (P1 pilin gene clone) or pBP500 (PAK pilin gene clone) plasmid (marked as GA1-200, GA1-500, GA2-200 and GA2-500) with anti-PAK polyclonal rabbit serum. [^{125} I] protein A was used to indicate the expressions of antibody-bound protein.

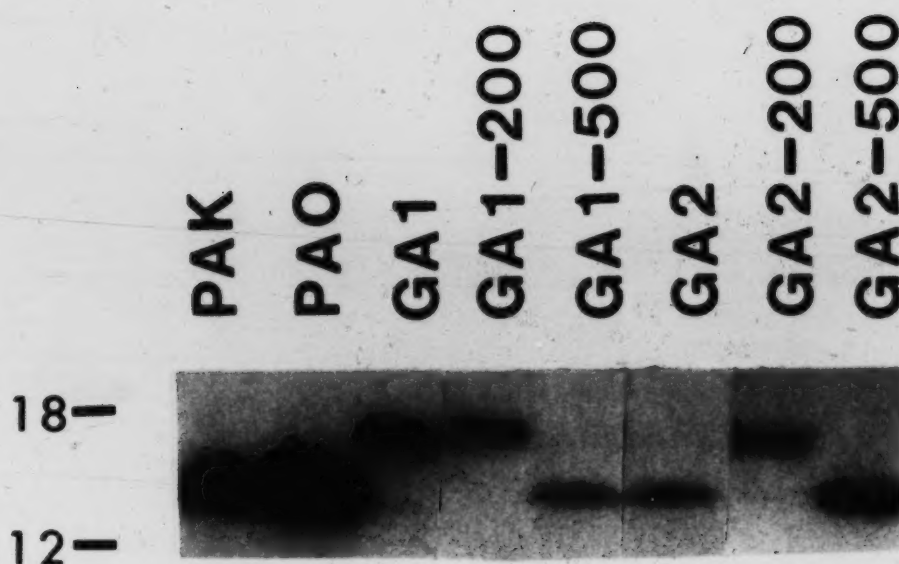


Fig.IV-12 Autoradiograph of an SDS-PAGE immunoblot showing reactivity of pilin expression in whole-cell bacterial lysates from eight strains of *P. aeruginosa*, PAK, PAO, GA1, GA2, GA1 or GA2 containing pBP200 (P1 pilin gene clone) or pBP500 (PAK pilin gene clone) plasmid (marked as GA1-200, GA1-500, GA2-200 and GA2-500) with anti-PAO polyclonal rabbit serum. [^{125} I] protein A was used to indicate the expressions of antibody-bound protein.

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CHAPTER V

COMPARATIVE STUDIES OF SEVERAL AMINO ACID SEQUENCES OF
PSEUDOMONAS AERUGINOSA PILINSA. INTRODUCTION

Pili are thin, non-flagella protein filaments found on the surface of many types of bacteria. Pseudomonas pili are known to play an important role in adherence and colonization of mammalian hosts, although the specific cell surface alteration that allows the adherence of P. aeruginosa has not been well defined.

Researchers have shown that pilus proteins promote several important functions. Specific regions of the polypeptide chain are responsible for anchoring pilin to membranes, transporting pilin across membranes, and promoting various subunit-subunit interactions. Moreover, surface-exposed regions of the pilus protein are usually immunologically active, or function as recognition sites for pilus-specific bacteriophages or mamalian cell receptors.

Several serologically related or unrelated isolates of P. aeruginosa pilin genes have been sequenced: PAK (Pasloske et al, 1985; Sastry et al, 1985), PAO (Sastry et al, 1985), CD (Pasloske et al, 1988a), P1 (Pasloske et al, 1988), K122-4 (Pasloske et al, 1988b), KB7 (Sastry, unpublished data), 492C

(unpublished data) and GA1&2 (GA) (this study). These eight pilin peptide sequences were analyzed by PC/GENE computer programs [PC/GENE: the nucleic acid and protein sequence analysis software system (C) A. Bairoch/University of Geneva; (TM) IntellGenetics Inc and Genofit SA]. The comparative studies of the characteristics of the pilin peptides, homology alignments, hydropathic indices, hydrophilicity profiles, secondary structure predictions and antigenicity predictions are presented in this chapter.

B. RESULTS

1. Characteristics of *P. aeruginosa* Pilin Proteins

As background information, PAK, PAO, CD, P1, K122-4, KB7, 492C and GA pilin protein sequences were analyzed by PC/GENE computer programs. The numbers of amino acid residues, molecular weight and theoretical isoelectric points are listed in Table V-1.

2. Amino Acid Compositions of *P. aeruginosa* Pilin Proteins

The amino acid composition of each pilin sequence is listed in Table V-2. Among all the pilin protein sequence compositions, there is no histidine in any pilin sequence. Most of the pilin proteins are very rich in amino acids Ala, Gly, Ile, Thr, Lys and rich in Leu, Ser, Val, except KB7. KB7 is very rich in Gln and has a low amount of Lys. Also, 492C has a higher composition of Arg and a lower composition of Thr.

3. Comparison of the Eight Pilin Sequences of *P. aeruginosa*

PAK, PAO, CD, P1, K122-4, KB7, 492C and GA(1&2) were aligned to obtain the greatest possible homology by the PC/GENE computer program "CLUSTAL". The results are shown in Fig. V-1. The program also generated a dendrogram (Fig.V-2) for all eight pilin sequences, which provides more information

of the homology among all the pilins.

Visual examination of Fig.V-1 revealed one highly conserved region (HC), four moderately conserved regions (MC-1, MC-2, MC-3 and MC-4) and three hypervariable regions (HV-1, HV-2 and HV-3) for all the pilin peptides. This information is shown in Fig.V-3.

4. Secondary Structure Predictions of the Eight Pilin Proteins

The secondary structures of PAK, PAO, CD, P1, K122-4, KB7, 492c and GA(1&2) were predicted by the PC/GENE computer program using the method of Garnier (Garnier *et al*, 1978). The results are shown in Fig.V-4. Almost all the pilin peptides have helical and extended structure potentials at their N-terminal and central regions. Most pilin proteins have strong extended, turn and coil structural tendency at the C-terminal region of the peptides.

5. Surface Profile and Antigenicity Predictions of Eight Pilin Peptides

All eight *P. aeruginosa* pilin protein sequences were analyzed by PC/GENE programs and the plots of their hydropathy index (Kyte and Doolittle, 1982) computations and hydrophilicity profiles (Hopp and Woods, 1981) are presented in Fig.V-5. Chain flexibility (Karplus and Schulz, 1985) and hydrophilicity profiles were the two parameters chosen to predict the antigenic sites of each pilin protein peptide. The

computed plots of these results are shown in Fig.V-6 and Fig.V-7.

The most flexible regions and the highest hydrophilic regions of the pilin peptides generated by the programs are listed in Table V-3 for comparison.

C. DISCUSSION

1. Primary and Secondary Structure Analysis

In Fig.V-1, the predicted amino acid sequences of the eight known *P. aeruginosa* pilins have been aligned to reveal the similarities information. The result showed that every pilin peptide has a short 6-7 residue leader sequence and 28 almost highly conserved residues at the N-terminus. There are also four moderately conserved regions found encompassing positions 29-61, 75-93, 116-148 and 160-173, while the remaining sequences located between positions 61-75, 93-116 and 148-160 were considered as hypervariable regions. The schematic representation of the locations of these regions is shown in Fig.V-3.

Fig.V-4 (A) and (B) show the hydropathic index computations and hydrophilicity profiles of all the pilin proteins. Examination of these plots revealed the extreme hydrophobicity of residues 1-28 in the N-terminal region of each pilin peptide. The fact that this region is so highly conserved among pili from different genera suggests it must have an important structural role. Studies by Watts *et al* (1983a) have shown that the two Tyr residues at positions 31 and 34 (Fig. V-1) are completely buried in intact pili, but completely exposed when the pili are dissociated into dimeric subunits with the non-ionic detergent octyl glucoside. These

observations provided compelling evidence that the end of the hydrophobic N-terminal region is involved in subunit-subunit interactions. Mattick et al (1987) have suggested that this region may play an important role in pili morphogenesis through interactions with accessory factors involved in the pilus assembly system. More recent studies have shown that only the first 36 amino acids of the prepilin are required for membrane integration and that the C-terminal region of propilin may associate with the N-terminal domain to stabilize the region between residues 30-65 against degradative process (Pasloske et al, 1988).

It is also worth noting that certain amino acids besides the hydrophobic stretch at the N-terminus appear to be conserved in all eight pilin proteins (Fig.V-1): Leu-50; Lys-51; Thr-52 and -133; Gly-84 and -142; Cys-147 and -165; Pro-162, and an aromatic amino acid at position 160. Several pilin proteins have characteristics which make them unusual: i) K122-4 has seven residues instead of six as a putative leader peptide; it is also the longest NMePhe pilin found in P. aeruginosa isolates studied so far, and it has two additional cysteines at positions 72 and 111. ii) KB-7 also contains four cysteines, two additional ones are at positions 57 and 72. It has a much higher composition of Gln than the other pilin proteins and a lower composition of Lys. iii) 492C has a higher composition of Arg than the other pilins and it has the highest pI of 10.26.

The predicted secondary structures were deduced by the method of Garnier (Garnier *et al*, 1978) It is suggested by the program designers that this method has higher accuracy than classical Chou & Fasman (1978) method. Four types of secondary structures are indicated in Fig.V-4: helical conformation, extended conformation, turn conformation and coil conformation.

It is of particular interest to find that the hydrophobic N-terminal region exhibits high potential for both helical and extended conformations. This may indicate that the amino-terminal portion of the molecule is unstable (Philips *et al*, 1979), and that its conformation may be governed by environmental conditions: helical in the assembled pilus and an extended structure during synthesis and transport into the membrane (Steiner *et al*, 1980; Briggs *et al*, 1986). In this model, transport would involve the hydrophobic N-terminal sequence undergoing a transition from extended structure to helical structure as it partitions into the membrane.

A second interesting feature of the NMePhe pilin structure is within the MC-1 region encompassed by regions around position 35-60, as shown in Fig.V-4. The secondary structure predictions indicate that this segment is largely helical. This suggests that a helical structure in this portion of the molecule may have a key function in pilin structure. Pasloske *et al* (1988c) have suggested that the first 55 residues of *P. aeruginosa* PAK pilin may partition

into the cytoplasmic membrane as a "helical hairpin" structure in which the positively charged leader sequence remains in the cytoplasm, residues 1-20 (or part there of) pass through the membrane as an α -helix domain, residues 21-34 form a loop structure in the periplasmic space, while the segment encompassing residues 35-55 passes back through the cytoplasmic membrane as an α -helix.

A third significant feature of NMePhe pilin structure is related to the cysteine residues and putative disulfide bridges. The cysteine residues in pilins from PAK and PAO are known to exist in the disulfide state (Sastry *et al*, 1985). Those cysteine residues existing in the other pilin proteins have not been examined to determine whether they are in the disulfide bridge configuration. It may be seen in Fig.V-1 that different configurations of putative disulfide bridges exist among the NMePhe pilins shown. Most of the pilins (PAK, PAO, CD, P1, 492C and GA1&2) contain a putative disulfide loop near the C-terminus. The number of amino acids between Cys residues is either 12 (PAK, PAO and CD) or 17 (P1, 492C and GA1&2); a second configuration is presented by KB7 pilin, it has a 15-residue putative disulfide loop at positions 57-72 (Fig.V-1) near the N-terminus and a 12-residue loop at the C-terminus. The third configuration, which contains both the large putative disulfide loop in the central region (positions 72-111, Fig.V-1) as well as the C-terminal loop, is presented by K122-4 pilin.

The dendrogram of the eight pilin sequence alignments displays a tree clustering of the protein sequences of PAK, PAO, P1, CD, K122-4, KB7 and GA(GA1&2) based on overall similarity (Fig.V-2). It revealed that the GA protein sequence has the highest homology to the sequence of 492c, CD sequence is mostly similar to the sequence of PAO.

2. Antigenicity Predictions of NMePhe Pilin Peptides

The antigenic regions of the *P. aeruginosa* PAK pilus protein were first determined by Watts *et al* (1983b) and Sastry *et al* (1985b) using an arginine-specific cleavage method and immunoblot experiments. One or more major epitopes were identified in the region 70-110, while weak epitopes were found in the N-terminus and C-terminal disulfide loop region. Recent studies with synthetic peptides have shown that PAK pilin has five surface regions encompassing positions 41-49, 58-71, 75-85, 108-117 and 130-140, including the disulfide loop region (Lee *et al*, 1989a, 1989b). Based on the above studies and the studies by Sastry *et al* (1985a) on natural peptides of PAK pilin, it is probable that the immunodominant epitopes of PAK pilin are located within the 75-85 and 108-117 amino acid sequences. More recent studies with native peptides from PAO1 pilin indicated that the immunodominant epitopes of this pilus type may reside in a strongly predicted β -turn between residues 78-82, and a putative "loop" region encompassing residues 104-110 (Sastry and Paranchych, unpublished results).

However, PAK and PAO are the only pilin proteins which have been studied to date by immunological experiments. Therefore, PC/GENE computer programs were used to predict the antigenic sites of each pilin.

In general, antigenic sites are defined as those residues of a native protein that are recognized by antibodies raised to a native protein, native protein fragments, or synthetic peptides. It is most likely that these sites are accessible or on the surface of a protein, and these regions are probably more mobile and hydrophilic than interior regions. Algorithms for flexibility and hydrophilicity have been used to predict antigenicity of the pilin proteins. Segmental flexibility seems to be correlated with the location of continuous epitopes. The computer-plotted surface profiles of eight pilin proteins calculated with the flexibility parameters and hydrophilicity parameters are presented in Figs.V-6 and V-7, the amino acid information of these regions is listed in Table V-3.

Inspection of these data showed that the general patterns of the two profiles are similar. However, the predicted antigenic regions indicated by the high flexibility or hydrophilicity varied with every parameter set. Generally, from the eight proteins investigated, the flexibility parameters provided more information of the regions having higher antigenicity than the hydrophilicity parameters. Since no information about the immunological properties of these

proteins exists, no comparison can be made. It was obvious that no single parameter set was able to predict all of the antigenic sites.

Hydrophilicity algorithms had been^o used extensively to select potential synthetic peptides from protein sequences capable of producing antipeptide antibodies which recognize linear antigenic determinants on the protein. Antibodies specific for synthetic peptides can be prepared by coupling this peptide to a protein carrier (Parker and Hodges, 1985). This will also promote the NMePhe based vaccine development. It has been reported that chemically synthesized peptides corresponding to two different regions of the VPI polypeptide of foot-and-mouth disease virus (FMDV) produced high levels of serotype-specific virus neutralizing antibody in cattle, guinea pigs and rabbits (Bittle *et al.*, 1982). Therefore, it is believed that the use of synthetic peptides as multi-valent vaccines will be successful in the near future (Paranchych, 1989). Further studies need to be carried out on the purified pilin proteins (CD, P1, K122-4, KB7, 492C and GA1&2), and immunological studies directed at determining the antigenic regions are also necessary.

Table V-1 Computed characteristics of several
Pseudomonas aeruginosa pilin proteins

Sequence name	Number of residues	Molecular weight	Theoretical isoelectric point
PAK	150	15622	8.80
PAO	149	15512	6.58
CD	150	15743	8.79
P1	154	16278	9.19
K122-4	157	16175	8.93
KB7	154	15719	6.31
492C	154	16554	10.26
GA(1&2)	154	16024	8.76

* Analyzed by PC/GENE computer program.

Table V-2 Amino acid compositions* of several
Pseudomonas aeruginosa pilin proteins

Amino acid	Pilin proteins							
	PAK	PAO	CD	P1	K122-4	KB7	492C	GA(1&2)
Ala(A)	20	19	17	18	25	20	18	17
Gly(G)	15	13	14	11	13	16	12	16
Ile(I)	12	15	15	14	11	10	13	15
Leu(L)	12	8	9	9	11	11	14	10
Met(M)	3	3	3	2	4	3	2	2
Cys(C)	2	2	2	2	4	4	2	2
Val(V)	8	10	9	12	7	11	9	8
Phe(F)	3	4	4	2	2	3	3	2
Pro(P)	7	6	5	6	5	6	7	6
Tyr(Y)	2	3	1	4	5	1	4	4
Trp(W)	2	1	1	2	1	2	3	2
Asn(N)	4	6	6	5	5	5	5	5
Gln(Q)	5	5	5	7	5	11	5	5
Ser(S)	8	10	9	13	9	11	12	13
Thr(T)	15	18	20	16	24	22	13	17
Arg(R)	3	3	3	3	3	5	8	3
Lys(K)	14	10	12	14	12	4	12	13
Asp(D)	8	6	4	7	8	6	6	8
Glu(E)	7	7	9	7	3	3	6	6
His(H)	0	0	0	0	0	0	0	0

* Analyzed by PC/GENE computer program.

Table V-3 List of antigenic regions* of each pilin predicted by flexibility and hydrophilicity parameters

Pilins	Regions predicted by parameters of	
	Flexibility	Hydrophilicity
PAK	2-8, 35-41, 47-54, 64-70, 84-90, 96-102 114-120 124-130, 136-142, 143-149	70-76 115-120 137-142
PAO	35-41, 47-54 62-68, 69-75, 72-78 82-88, 97-103, 110-116 134-140, 143-149	50-55, 54-59 83-88
CD	35-41, 47-54, 52-58, 61-67 72-78, 82-88, 97-103 110-116, 124-130, 134-140	83-88, 97-102 116-121
P1	2-8, 35-41, 57-63, 67-73 78-84, 85-91, 95-101 109-115, 123-129, 134-129	95-101, 98-103 124-129
K122-4	50-56, 58-64, 75-82, 88-94 95-101, 114-120, 126-132 137-143, 145-151, 150-156	51-56, 74-80 138-143
KB7	27-33, 34-41, 57-63, 74-80 85-91, 91-97, 104-110 130-136, 147-154	36-41, 58-63 144-149
492C	2-8, 35-41, 58-64 65-71, 78-84 84-90, 89-95, 110-116 123-129, 135-141	67-73, 70-75 124-129
GA162	2-8, 58-64, 67-73, 78-84 85-91, 95-101, 103-109 109-115, 123-129, 135-141	93-98, 95-100 124-129

* Data were produced by PC/GENE computer program

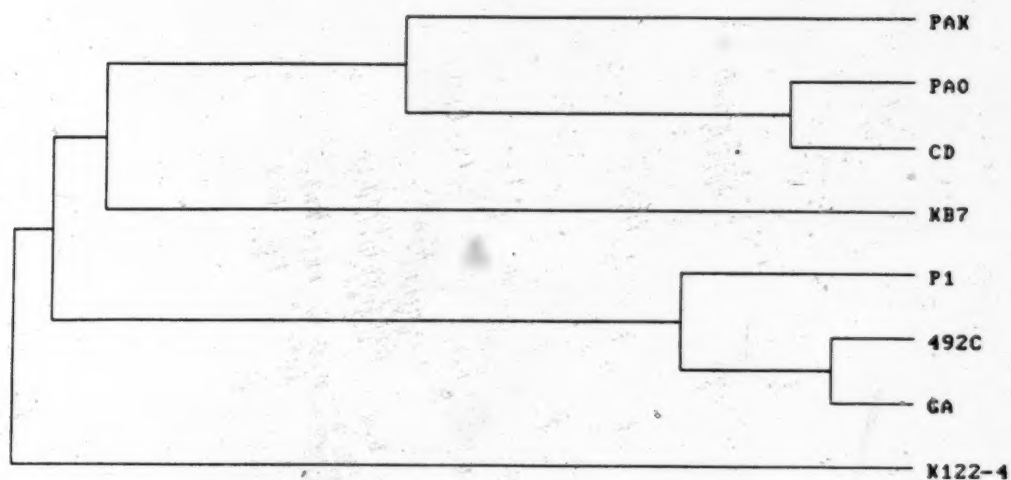
PAK	MKA-QKGFTLIELMIVVAIIIGILAAIAIPQYQNYVARSEGASALASVNPL	49
PAO	MKA-QKGFTLIELMIVVAIIIGILAAIAIPQYQNYVARSEGASALATINPL	49
CD	MKA-QKGFTLIELMIVVAIIIGILAAIAIPQYQNYVARSEGASALATINPL	49
P1	MKA-QKGFTLIELMIVVAIIIGILAAIAIPQYQDYTARTQVTRAVSEVSAL	49
KB7	MKA-QKGFTLIELMIVVAIIIGILAAIAIPQYQNYVSRQVSRVMAEAGSL	49
492C	MKA-QKGFTLIELMIVVAIIIGILAAIAIPQYQDYTARTQVTRAVSEISAL	49
GA	MKA-QKGFTLIELMIVVAIIIGILAAIAIPQYQDYTARTQVTRAVSEISAL	49
K122-4	MKAAQKGFTLIELMIVVAIIIGILAAIAIPAYQDYTARAQLSERMTLASGL	50
	*** ***** * *	

PAK	KTVEEALSRGWSV-KSGTG--TEDATKKEVPLGVAADANKLGT-----I	91
PAO	KTVEESLSRGIAGSKIKIG--TTASTATETYVGVPEPDANKLGV-----I	92
CD	KTVEESLSRGIAGSKILIG--TTASTADTTYVGIIDEKANKLGT-----V	92
P1	KTAESAILEGKEIVSS-----ATPKDTQYDIGFTESTLLDGS--GKSQ	91
KB7	KTAVEACLQDGRITAVGTAAGQCDPGATGSSLLTGASQTSQTLP--TNTGV	97
492C	KTAESAILEGKKLVS-----DSPKNDEYDLGFTSSTLLTG--ELRGR	91
GA	KTAESAILEGKKLVS-----DTPGNNEYDLGFTSSTLLTG--SGKGC	91
K122-4	KTKVSDIFSQD-----GSCPANTAATA---GIEKDTDINGKYVAKV"	89
	** * . . . *	

PAK	ALKPDPADGTADITLFTMGAGPKNKGKIITLRTAAD-G--LWKCTSD	138
PAO	AVAIE-DSGAGDITFTFQTGTSSPKNATKVITLNRTA-D-G--VWACKST	137
CD	AVTIK-DTGDGTVKFTFATGQSSPKNAGKEITLNRTA-E-G--VWTCTST	137
P1	IQVTDNKDGTVELVATLGKSSGSAIK-GAVITVSRK-ND-G--VWNCKIT	136
KB7	PQVLDPLTTQTTHIATFGNGASAAIS-GQTLTWTRD-VNGG--WSCATT	142
492C	SRLTKLIPQLRRFLVPWAASSGKGIA-GAVITVKRD--DKG--VWTCGIT	136
GA	IKIDKADTATPEISGTLGNSSGKGIA-GAVITVKRD--DKG--VYTGGIT	136
K122-4	TGGTAAASGGCTIVATMKASDVATPLRGKTLTLTLGNADKGSYTWACTSN	139
	 * . . . *	

PAK	QDEQ-----FIPKGCSC-----	150
PAO	QDPM-----FTPKGCDN-----	149
CD	QEEM-----FIPKGCNK-----P	150
P1	KTPTAWKPNYAPANCPK-----S	154
KB7	VDAK-----FRPNGCTD-----	154
492C	GSPTNWKANYAPANCPK-----S	154
GA	GSPTDWKTNWAPANCPK-----S	154
K122-4	ADN-----KYLPKTCQTATTTTP	157
	 *	

Fig.V-1 Comparison of the eight pilin protein sequences of *Pseudomonas aeruginosa*. The pilin sequences were analyzed and aligned by PC/GENE computer program. "*" shows a perfectly conserved position, "." shows a well conserved area. The numbers above the alignment sections indicate the total number of positions including spaces rather than a particular pilin sequence or amino acid.



Dendrogram of the alignment.

Fig.V-2 Dendrogram of the pilin sequence alignments of *Pseudomonas aeruginosa* strains of PAK, PAO, P1, CD, K122-4, KB7, GA1&2.

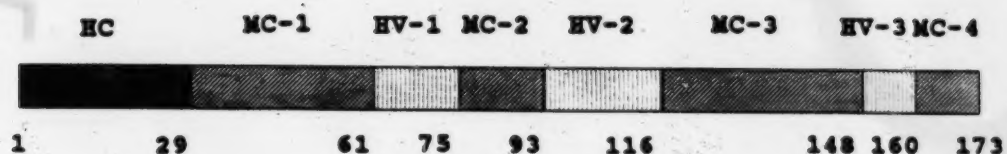
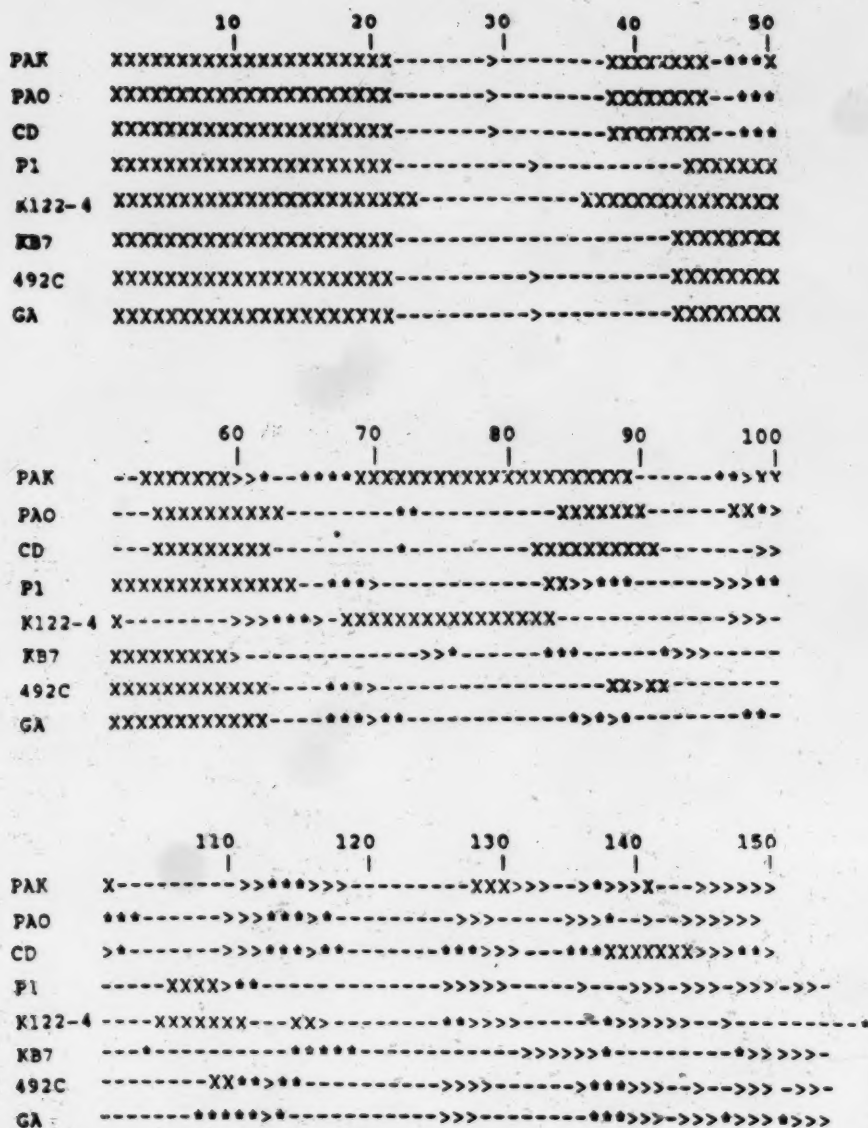


FIG.V-3 Schematic representation of the eight pilin peptides. One highly conserved region is shown as HC, four moderately conserved regions are marked as MC-1, MC-2, MC-3 and MC-4, three hypervariable regions are marked as HV-1, HV-2, HV-3. The numbers indicate the total number of positions including spaces which are shown in Fig.V-1.



Helical conformation: X
Turn conformation: >

Extended conformation: -
Coil conformation: *

Fig.V-4 Eight *Pseudomonas aeruginosa* pilin protein secondary structure predictions by the method of Garnier.

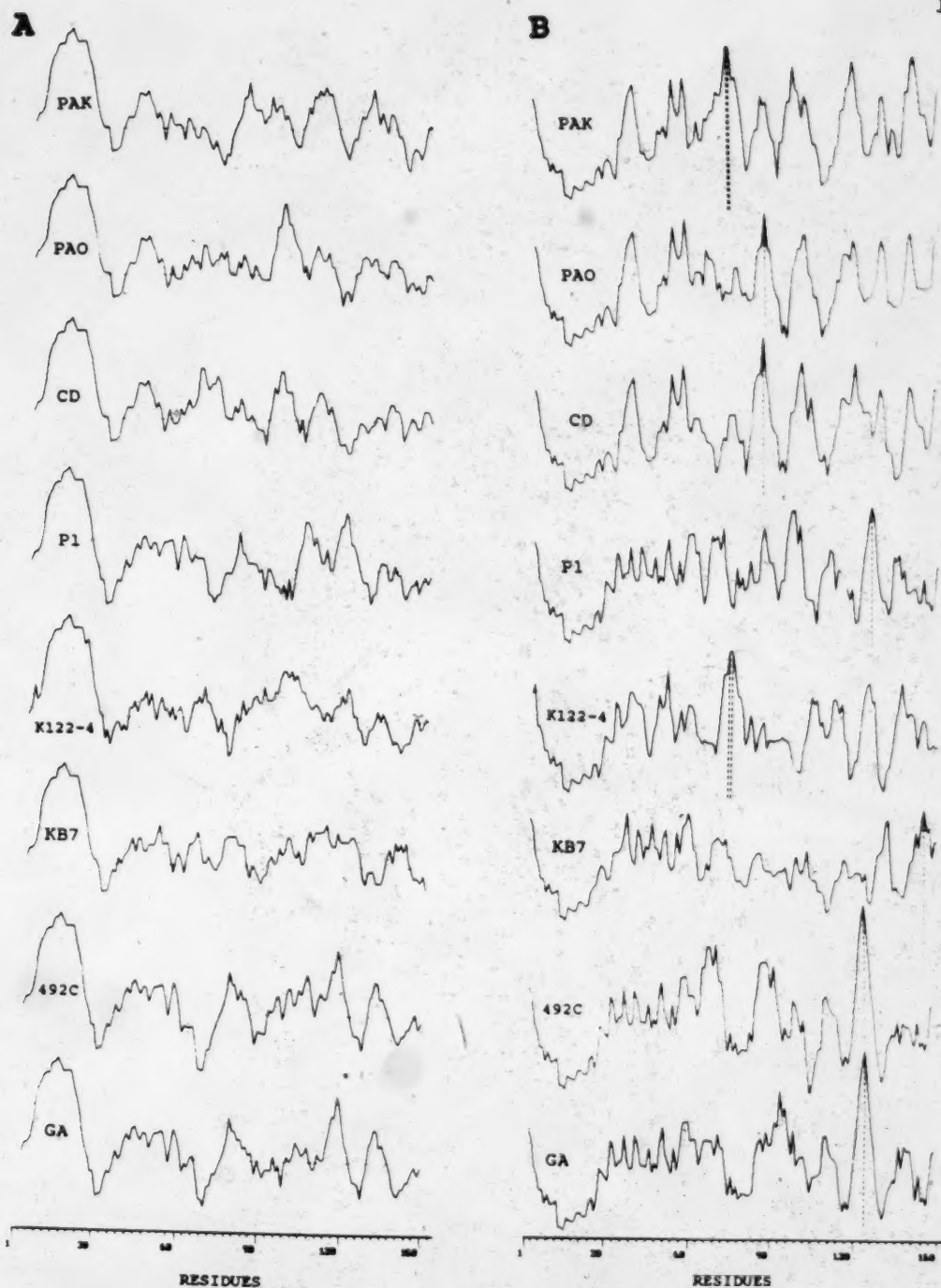


Fig.V-5 Hydropathic index computations (A) and hydrophilicity profiles (B) of different pilin protein sequences analyzed by the method of Hopp and Woods (Hopp and Woods, 1981). Upper line regions are hydrophobic, under line regions are hydrophilic. Vertical dotted lines indicate the highest points of hydrophilicity of different pilin proteins.

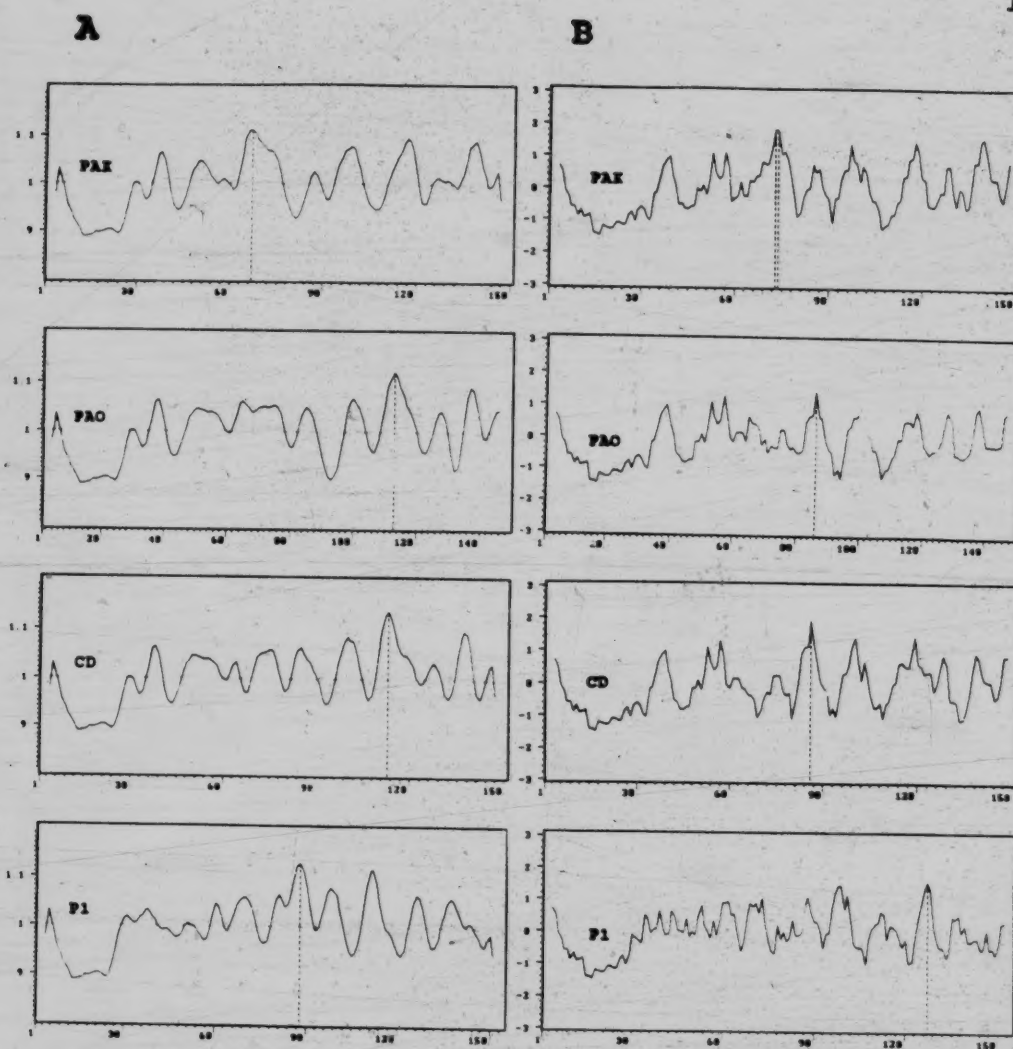


Fig.V-6 Surface profile predictions of PAK, PAO, CD and P1 pilins by Karplus & Schulz flexibility parameters (A) and Hopp & Woods hydrophilicity parameters (B). The X axis value represents residue number of the peptide. The Y axis value is B[norm] in (A) and average hydrophilicity value in (B). Vertical dotted lines indicate the highest points of flexibility (A) and hydrophilicity (B) of different pilin proteins.

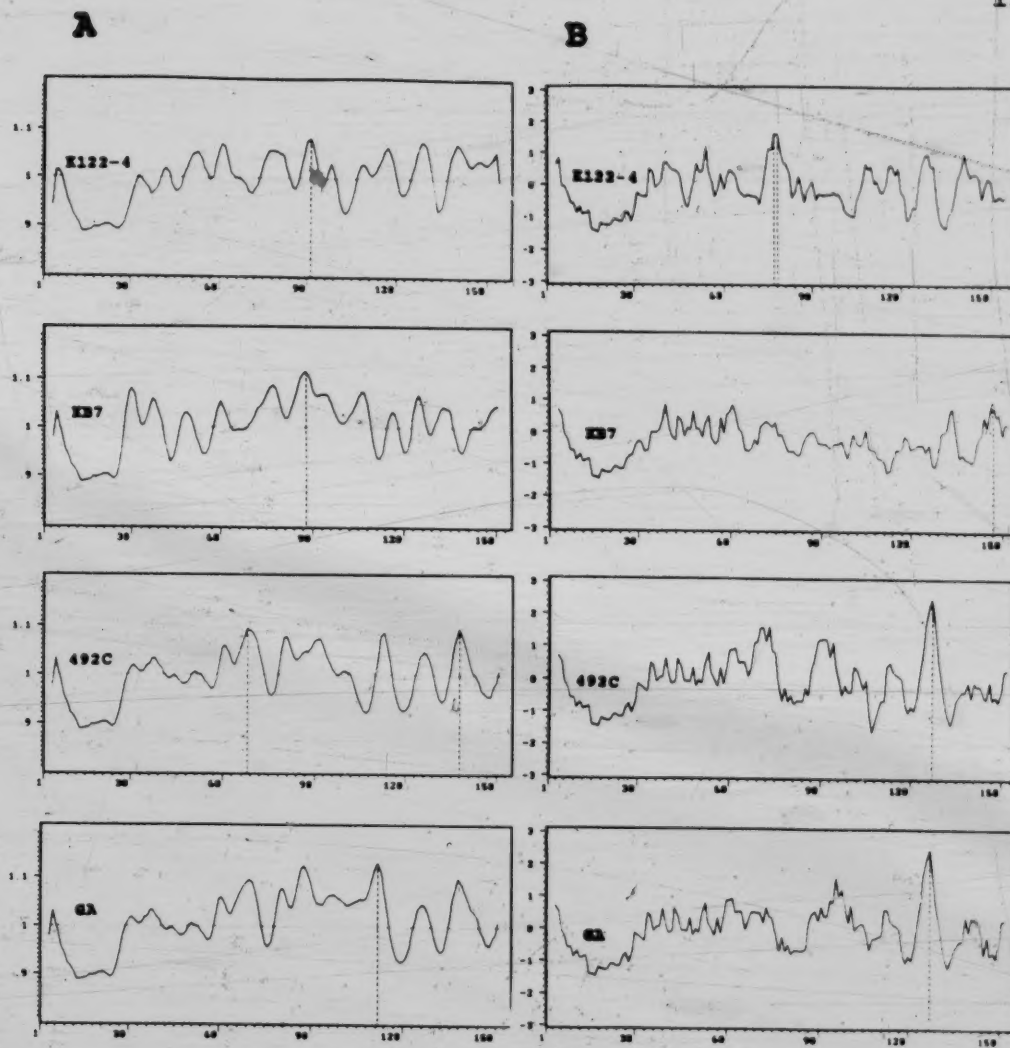


Fig.V-7 Surface profile predictions of K122-4, KB7, 492C and GA1&2 pilins by Karplus & Schulz flexibility parameters (A) and Hopp & Woods hydrophilicity parameters (B). The X axis value represents residue number of the peptide. The Y axis value is B[norm] in (A) and average hydrophilicity value in (B). Vertical dotted lines indicate the highest points of flexibility (A) and hydrophilicity (B) of different pilin proteins.

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